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"ANALYSIS AND CHARACTERIZATION OF *DE NOVO* CENTRIOLE BIOGENESIS IN ACENTRIOLAR *DROSOPHIL*A SAS-4 -/- CELLS"

Dissertação de Candidatura ao grau de Mestre em Oncologia submetida ao Instituto de Ciências Abel Salazar da Universidade do Porto.

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“Life begins at the end of your comfort zone”

Anonymous

Para os meus Pais, Emília e Carlos

À Olga Afonso

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Por vezes, pequenos elementos mudam o rumo das nossas vidas.

No meu caso, esse elemento foi um simples envelope com um conjunto de cartas deixado, em meados de Outubro do ano 2011, na secretaria de um instituto científico localizado na cidade do Porto.

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Abstract

Centrioles, the key elements on centrosome biogenesis and function, are replicated during S phase of each cell cycle. Two main mechanisms of centriole assembly have been described: 1) a template or canonical, and 2) a *de novo*. While the template mechanism has been assigned as the major mode of centriole assembly in somatic cell divisions, the nature of the *de novo* mechanism of centriole assembly is less well understood, especially in proliferating somatic cells. Moreover, the function of the mother centriole as a template in the centriole duplication process has been recently brought into question (Rodrigues-Martins et al., 2007b). Here, we show that expression of DSas-4-GFP in somatic acentriolar DSas-4 mutant *Drosophila* cells led to *de novo* centriole formation. Mitotic DSas-4 rescued cells showed amplification and co-localization of *de novo* DSas-4-GFP foci with the centriolar and PCM proteins DSas-6, Ana1, D-PLP, Asl and Cnn, respectively. Live cell imaging analysis showed that the newly formed DSas-4-GFP foci nucleate discrete astral microtubule and mitotic spindle assembly seemed to occur through an outside-inside process. Moreover, we rescued the expression levels of the centriole-specific proteins Asl and DSas-6, suggestive of an activation of the centriole biogenesis pathway. Electron microscopy analysis revealed the presence of centriole-like structures as well as clouds of electron-dense material lacking centrioles.

This study reinforces the notion that *de novo* and template mechanisms of centriole assembly may be variations of a common pathway based on the same molecular machinery. Centriole biogenesis is a template-free process in which the mother centriole may function as a platform for regulatory proteins involved in the centriole duplication process whereby offering an ideal environment for centriole duplication. Therefore, control of centriole number, spatial restriction along with a kinetic advantage might be the key factors that the mother centriole offers to the centriole biogenesis process.

Key words: centrioles; centrosomes; *de novo* centriole biogenesis; DSas-4 protein; Pericentriolar material; *Drosophila* Sas-4 mutant cell line

Resumo

Os centríolos, elementos chave na formação e funcão do centrosoma, são replicados durante a fase S de cada ciclo celular. Existem dois principais mecanismos de duplicação: 1) através de um molde, e 2) o *de novo.* Enquanto que o mecanismo molde tem sido reconhecido como o principal modo de formação de centríolos nas células somáticas em divisão, a natureza do modo *de novo* é ainda pouco compreendida, principalmente durante a proliferação de células somáticas. Além disso, o papel do centríolo mãe como modelo no processo de duplicação dos centríolos foi recentemente questionado (Rodrigues-Martins et al., 2007b). O presente estudo mostra que a expressão da proteína DSas-4-GFP em células acentriolares de *Drosophila* mutantes para a proteína DSas-4 leva à formação *de novo* de centríolos. Células mitóticas a expressar DSas-4-GFP mostram amplificação e co-localização dos foci de DSas-4-GFP com as proteínas centriolares e centrosomais DSas-6, Ana1, D-PLP, Asl e Cnn, respectivamente. Análise através de microscopia em células vivas revelou que os foci de DSas-4-GFP são capazes de gerar feixes de microtúbulos astrais e a formação do fuso mitótico parece ocorrer através de um processo de fora para dentro. Adicionalmente, foi verificado um aumento nos níveis de expressão das proteínas especificamente associadas com os centríolos, Asl and DSas-6, o que poderá sugerir uma re-activação do processo de formação de centríolos. A análise por microscopia electrónica revelou a presença de estruturas similares a centríolos, bem como locais com material pericentriolar sem centríolos.

Este estudo reinforça a ideia de que os processos *de novo* e molde são potencialmente variações de um mesmo processo que tem por base a mesma maquinaria molecular. A biogénese dos centríolos é um processo que não necessita de um molde, e em que o centríolo mãe poderá funcionar como uma plataforma para proteínas reguladores involvidas na biogénese de centríolos, assegurando um ambiente ideal para a duplicação dos centríolos. Assim, o controlo do número de centríolos, restrição espacial e uma vantagem cinética poderão ser os factores principais que o centríolo mãe oferece ao processo de biogénese de centríolos.

Palavras-Chave: centríolos; centrosoma; biogénese *de novo* de centríolos; proteína DSas-4; material pericentriolar; linha celular mutante *Drosophila* Sas-4

Abbreviations

aMTOCs – acentriolar microtubule organizing centres
Ana1-3 – anastral spindle 1-3
Asl – asterless
BSC-1 –African green monkey kidney epithelial
CDK-2 – cyclin-dependent kinase 2
CDK5RAP2 – CDK5 regulatory subunit-associated protein 2
CENPJ – Centromere protein J
CEP135 – centrosomal protein
CEP152 – centrosomal protein
CEP192 – centrosomal protein
CHO – Chinese hamster ovary
Cnn – Centrosomin
CP110 – Centriolar coiled-coil protein
CPAP – centrosomal P4.1- associated protein
D-PLP – Drosophila pericentrin-like protein
DSas-4 – *Drosophila* spindle assembly abnormal 4
DSas-6 – *Drosophila* spindle assembly abnormal 6
GDP – guanosine diphosphate
GTP – guanosine triphosphate
hSAS-6 – human spindle assembly abnormal 6
LECA – last eukaryotic common ancestor
MTOC – microtubule organizing centre
MTs – microtubules
NAB – nuclear associated body
PCM – Pericentriolar material
PLK1 – polo-like kinase 1
PLK4 – polo-like kinase 4
S2R+ - S2 receptor plus
SAS-4 – spindle assembly abnormal 4
SAS-5 – spindle assembly abnormal 5
SAS-6 – spindle assembly abnormal 6
SPB – spindle pole body
SPD-2 – spindle defective 2
STIL – SCL/TAL1 interrupting locus

TCP10 – T-complex protein 10

γ-TuRCs – γ-tubulin ring complexes

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1. INTRODUCTION

1. Introduction

"Omnis cellula e cellula" was postulated two centuries ago by the German physician Rudolf Virchow and it was the first approach defining that every cell arises from a preexisting parent cell. Back to our century, it is now known that Virchow's definition is undoubtedly correct and that cells need to proliferate to ensure progeny and the continuity of life (Rieder, 2006, pp.439).

Cell division is the result of a complex and strictly regulated process composed by distinct stages integrated in the cell cycle. In eukaryotes, the major goal of the cell cycle is to reproduce a daughter cell inheriting the same diploid number of chromosomes from the mother cell in a highly dynamic process called Mitosis. After DNA replication in interphase, sister chromatids of each chromosome have to be segregated to assure the euploidy of the daughter cells during mitosis. Therefore, an important biological machine was developed by the cells in order to organize chromosomes during metaphase and to accurate segregate them in anaphase - the Mitotic Spindle. The mitotic spindle is a symmetrical, dynamic and bipolar structure made up of microtubules (MTs) that interact with chromosomes via their kinetochores. Its assembly is orchestrated by centrosomes which are defined as the major microtubule-organizing centre (MTOC) in animal cells. Apart from its role in cell division, the centrosome is also a very important organelle in cell shape, polarity, motility, signaling, protein trafficking and in cilia/flagella formation (Bettencourt-Dias, 2013; Nigg, 2007; Nigg and Raff, 2009)

The advent of electron microscopy revealed the beautiful composition of the centrosome. This organelle is composed by a pair of barrel-shaped bodies called centrioles that are surrounded by an area of dense protein matrix termed the pericentriolar material (PCM) (Avidor-Reiss and Gopalakrishnan, 2013; Azimzadeh and Marshall, 2010; Debec et al., 2010; Nigg, 2007). As centrioles are at the basis of centrosome formation, it demands strict control of centriole number to ensure a correct number of centrosomes, and faithful chromosome segregation in mitosis. Indeed, it has been recognized the direct association between centriole number deregulation, centrosome overexpression and cancer (Nigg and Raff, 2009). Over the past decade, the mechanisms underlying centriole biology have been uncovered at a tremendous speed due to the development of more advanced imaging techniques and its combination with biochemical and cell biological approaches. A deeper knowledge of centriole structure, function and biogenesis is of major importance to a better understanding of centrosome biology and its role in disease.

2. Dissecting Centrosome and Centrioles

2.1. History and Inter-relationship

The centrosome was discovered by Boveri and Van Beneden in the late 1800s and it was coined as "the organ for cell division" with an important role in karyokinesis/mitosis and cytokinesis. Since that very first description, there was a long latency period in which research on centrosomes/centrioles languished. However, in the 80s and 90s, genetic and proteomic studies allowed the identification of specific centrosome and centriole proteins, as well as electron microscopy revealed the amazing architecture of centrioles within centrosomes.

Centrioles, which are the core components of the centrosome, are among the most conserved organelles through evolution in eukaryotic cells, having been lost in several lineages such as yeast, some amoebas and seed plants (Bettencourt-Dias, 2013; Carvalho-Santos et al., 2011). These organelles can also be converted into basal bodies when tethered to the cell membrane to template the axoneme required in cilia and flagella formation, indicating that centrioles are not only important structures in cell division, as they also perform a role in cell motility and signaling.

The evolutionary history of centrioles and centrosomes has just started to emerge. Recently, two phylogenetic studies (Carvalho-Santos et al., 2010; Hodges et al., 2010) unveiled the evolution of the centriole through the analysis of its protein components, from the centriole assembly machinery to the ciliary related proteins. Namely, Hodges and colleagues (2010) brought some important data supporting a previously held idea about centriole ancestral function, in which centrioles and centrosomes have not always coexisted during evolution. By analyzing the phylogenetic distribution of core centrosomal proteins across six major groups of eukaryotes, they found that the majority of the centrosomal proteins were restricted to Holozoa[1], suggesting a scenario in which the animal centrosome functioning as a MTOC in cell division is an holozoan innovation, which implies that in the temporal scale of evolution, centrioles could have been present before centrosomes, performing an ancestral role only as basal bodies (in motility/sensory function and cell organization) (Bornens and Azimzadeh, 2007). Interestingly, the occurrence and requirement of centrioles seems to be strongly correlated with the presence of flagella/cilia rather than with the centrosome (Carvalho-Santos et al., 2011; Hodges et al., 2010). It is known that many non-flagellated species, like some amoeba and fungi are able to produce modified MTOCs with no centrioles, such as the nuclear associated body (NAB) (amoebas) and the spindle pole body (SPB) (fungi) (Bornens and Azimzadeh, 2007; Debec et al., 2010). These data suggest that centrioles are mostly

[1]described as Metazoa and the choanoflagellate *Monosiga brevicollis*

essential in axoneme nucleation rather than in centrosome formation. It is possible to speculate that, during evolution, the ancestral centriolar structure only took part in cell division as a parsimonious solution to efficiently control the equal chromosome segregation into the daughter cells, providing a selective advantage to primitive eukaryotic cells (Bornens and Azimzadeh, 2007). Furthermore, Carvalho-Santos et al. (2010) have shown that centriole biogenesis is controlled by an evolutionary conserved and ancestral protein module (UNIMOD) that might have emerged in LECA (last eukaryotic common ancestor), and its occurrence is correlated with presence or loss of centrioles.

2.2. Role in Spindle Assembly during Cell Division

A controversial question in centrosome biology has been to what extent the centrosome is an essential and mandatory organelle for cell division, specifically acting as an MTOC needed for mitotic spindle formation and correct chromosome segregation during mitosis in animal cells. In contrast to what was believed at the time of the first pioneering studies on the centrosome, it is now known that the centrosome is not an essential organelle for cell division and survival. As described above this organelle was naturally lost in some groups of the eukaryotic tree of life such as in some species of fungi (ex. *Schizosaccharomyces pombe*). However, even those species organize the microtubule cytoskeleton using different MTOCs which do not contain centrioles, as exemplified by the spindle pole body in *S. pombe* and *Saccharomyces cerevisiae* (Bettencourt-Dias, 2013; Bornens and Azimzadeh, 2007; Debec et al., 2010). This is indicative that an MTOC does not need to be necessarily a centrosome, whereas a centrosome is always an organelle responsible for MT nucleation and anchoring.

In animals, the most documented and thoroughly studied case of a natural absence of centrioles is in female meiosis. In this specific event, as in the case of humans, the oocyte looses centrioles during oogenesis (in some point before metaphase of meiosis I) retaining just the PCM, whereas the spermatocyte does not retain the PCM, but an incomplete centriole pair. A fully functional and intact centrosome will only born after fertilization by the combination of the sperm-derived centrioles and the PCM supplied by the egg (reviewed in Debec et al., 2010). In animal somatic cells, centriole loss or inactivation was reported in differentiated cells, such as myotubes (muscle cells), in certain types of epithelia cells such as *Drosophila* wing epidermal cells and, also in neuronal cells (Bartolini and Gundersen, 2006; Bettencourt-Dias, 2013; Cunha-Ferreira et al., 2009; Debec et al., 2010). Surprisingly, a striking case of natural centrosome inactivation occurs in *Drosophila* cells, in which the interphase MT cytoskeleton is not nucleated by the centrosome, which seems to be only active as MTOC during mitosis (Rogers et al., 2008). In these last cases, centriole inactivation or loss is coupled with the

existence of non-centrosomal MT arrays, and even in cells that normally have functional centrioles, those centrioles are not obliged to take part in cell division within the context of the centrosome. This characteristic has been proven over the last years by a series of experiments taking advantage of specific antibodies that disrupted centrioles or laser irradiation to ablate the centrosome, in which it was demonstrated the dispensability of centrioles in cell division (Bobinnec et al., 1998; Khodjakov et al., 2000; Mahoney et al., 2006). These studies imply that additional non-centrosomal mechanisms of MT nucleation must exist, even in cells that naturally contain functional centrosomes.

Two major centrosome-independent pathways used for spindle assembly were described. A chromatin pathway that generates and stabilizes microtubules in the vicinity of the chromosomes, facilitated by a RanGTP gradient centered around chromosomes that triggers the release of TPX2 that, together with γ-tubulin complexes, nucleate MTs (reviewed in Meunier and Vernos, 2012). The CPC complex (INCENP, Survivin, Borealin and Aurora B) might also be involved in MT assembly in the periphery of chromatin (Moutinho-Pereira et al., 2013; Sampath et al., 2004). Microtubules are also nucleated from preexisting microtubules through the action of the Augmin complex in *Drosophila* (HAUS in human cells) that associates and recruits γ-TuRCs to assemble MTs, promoting microtubule nucleation and amplification (reviewed in Meunier and Vernos, 2012). It is noteworthy that these alternative acentrosomal MT nucleation pathways are not backup mechanisms, but can coexist with the centrosomal pathway for the successful mitotic spindle assembly and chromosome segregation (Debec et al., 2010; Meunier and Vernos, 2012; Moutinho-Pereira et al., 2013). Moreover, the Golgi and the nuclear envelope may also participate in MT nucleation, although the mechanism for MT assembly from both pathways has not yet been clarified (Bettencourt-Dias, 2013; Efimov et al., 2007; Meunier and Vernos, 2012). Interestingly, the evidence of centrosome dispensability in cell division was in stark contrast with reports of its requirement for cell cycle progression from interphase to mitosis in mammalian cells. Hinchcliffe et al. (2001) proposed the potential existence of a "centriole-dependent checkpoint" to monitor the G_1-S transition, since the microsurgical removal of centrosomes from BSC-1 (African green monkey kidney cells) cells right before S phase led to a G_1 arrest and blocked entry into S phase after the completion of a first cell cycle (Hinchcliffe et al., 2001). Nevertheless, this hypothesis was later refuted because the previous arrest in interphase most likely reflected a stress response rather than a specific novel checkpoint (Uetake et al., 2007).

Although centrosomes can be viewed as dispensable "facilitators" that help in many aspects of cell´s life, there are some situations in which they seem to be strictly required. Early embryonic divisions (e.g. syncytial mitosis in *Drosophila*), asymmetric cell divisions and cilia/flagella formation are the three main cases in which the centrosome is

just not a "facilitator", but rather, an indispensable organelle for the successful and normal organism development (reviewed in Nigg and Raff, 2009 and Debec et al., 2010).

2.3. Acentriolar Organisms and Cell lines

Centriole removal in somatic cells by micromanipulation, laser ablation or antibody injection allowed to infer that centrosomes are not essential to drive bipolar spindle assembly during mitosis. Despite the valuable conclusions of the last studies, they did not allow to track the acentriolar-induced cells over many generations and infer about their fate. In fact, there was still an intriguing question that remained to be decipher. *What would be the effect of centrosome removal in the development of a whole organism?.*

The model system *Drosophila melanogaste*r has been intensively explored in the last years to address that question. Basto *et al.* (2006) successfully developed an acentriolar lineage of flies through the insertion of a P element that truncated a specific centriole assembly component (*Drosophila* SAS-4 or DSas-4). These mutant flies (DSas-4^{S2214}), derived from heterozygous females, were able to progress through the first embryonic divisions owing to the maternal DSas-4 protein supply, and centrioles were lost as a result of the successive DSas-4 dilution over cell divisions ensuring the lack of a functional centrosome. Mutant flies were able to reach the adult stage in normal timing and morphology. Nevertheless, DSas-4^{S2214} mutants were highly uncoordinated and unfertile as a consequence of the lack of cilia in type 1 mechanosensory neurons and in sperm. Mitosis duration was slightly increased by 30-40 % in mutant cells as well as 30 % of dividing mutant neuroblasts produced two daughter cells of equal size or failed cytokinesis, although the development or the neuronal organization was not compromised. This study implies four major conclusions: (1) centrosomes/centrioles are not essential organelles in *Drosophila* development; (2) centrioles are essential for fly survival due to its role in cilia and flagella formation and not as an MTOC; (3) centrosomes have an important role in fly asymmetric cell divisions, and (4) centrosomes might promote the fidelity and favour the kinetics of cell division.

In *Drosophila*, the first cell line constitutively lacking centrioles (1182-4) was reported by Alain Debec and colleagues in 1982, although its origin still remains unclear (Debec, 1982). Recently, the same group has established and characterized new centriole-free *Drosophila* cell lines derived from homozygous *Drosophila* embryos for the mutation DSas-4 (Lecland et al., 2013). These cells exhibit a typical phenotype, in which the mitotic spindle usually presents broad poles with no recruitment of centrosomal and centriolar components such as D-PLP, Cnn, γ-tubulin and DSas-4. As in acentriolar *Drosophila* flies, mitosis is delayed and lasts nearly three times compared to wild-type cells, in which the majority of acentriolar cells particularly spend more time in prophase.

Live imaging analysis and MT regrowth experiments revealed that the mitotic spindle is built from discrete foci close to chromatin by an "inside-out" process, and there is the absence of centrosomal asters (Lecland et al., 2013). Concomitantly, the establishment of vertebrate acentriolar cell lines was successfully performed in DT-40 (hyperrecombinogenic chicken B cell line) cells by disruption of the centriole assembly proteins CEP152 and STIL. Both cell lines lack intact centrioles and show acentriolar MTOCs (aMTOCs) composed of satellite clumps of PCM components (e.g. γ-tubulin) with residual MT nucleation activity, but unable to duplicate. Also, mitotic spindles are formed by the chromatin or augmin-dependent pathways and are characterized by displaying a disorganized and unfocused MT array. Furthermore, mitosis timing in acentriolar DT-40 cells is higher compared to wild-type cells, in which G_2/M phases take more time to be accomplished. Interestingly, 30% of these cells exhibit a tendency to missegregate chromosomes during anaphase, originating an aneuploid state or even chromosome instability (CIN) events (Sir et al., 2013). Strikingly, a slight increase in aneuploidy was also reported in DSas-4^{S2214} mutant flies (Basto et al., 2006). The features of these acentriolar invertebrate and vertebrate cell lines revealed that functional centrosomes are important eukaryotic organelles to ensure a normal mitotic timing and to promote fidelity in mitosis, which might be predominantly important features in organisms with high number of chromosomes. It would be particularly interesting to address the effects of centriole loss on vertebrate development, although the challenge of this work relies on the higher complexity of vertebrates relatively to invertebrates, and therefore the necessity of this organelle in important functions such as responding to extracellular signals and in organ development.

Lastly, recent reports addressed the question of centriole loss in model systems that usually contain centrioles, in which centriole function was successfully disrupted through genetic approaches. Since there was no reports of centriole absence during the entire development of an organism, the natural absence of centrioles in metazoans seemed to be confined to specialized cells such as female oocytes. The flatworm *Planaria* was the first metazoan revealing that centrioles can be absent from proliferating cells and they are not essential for the normal development of this organism at any stage. An elegant study conducted by Azimzadeh et al. (2012) on the planarian specie *Schmidtea mediterranea* showed that centrioles are restricted to multiciliated cells and are absent from the only two types of non-ciliated cells able to divide in planarians: embryonic cells and neoblasts (a population of totipotent stem cells). The silencing of critical proteins involved in centriole duplication (SAS-4 and PLK4) through RNAi (RNA interference) led to a defect in the gliding locomotion of these species, as a consequence of impaired ciliary function, whereas the same depletions did not affect tissue regeneration ability.

Interestingly, it was also shown that planarians did not retain a subset of specific centrosomal proteins (SPD-2/CEP192, Cnn/CDK5RAP2 and Nek2) present in humans and in *Drosophila* genome, suggesting that centrosome loss during evolution was in parallel with the loss of the previous centrosomal proteins subset and, probably, also with a change in the pattern of embryonic cleavages (Azimzadeh et al., 2012). This study represented an important step for a better understanding of centrosome and centriole function during evolution and in organism development, demonstrating that centrioles can be retained only to produce cilia and are dispensable as centrosomes in cell division. It is likely that the presence of centrosome is more related with the need of this organelle to coordinate specific developmental processes, rather than an essential cellular requirement.

3. The Centrosome

3.1. From what is made a Centrosome ?

The centrosome is a non-membrane bound organelle composed of a centriole pair surrounded by a proteinaceous scaffold containing a large number of proteins referred as pericentriolar material (PCM) (Figure 1). Since centrosomes are central players in MT nucleation and organization, many cellular activities including cell motility, polarity, shape, cell division, transport of vesicles and targeting of signaling molecules are performed by this organelle. Centrosome is a dynamic cell component whose size is tightly regulated along the cell cycle. In interphase, it is usually small and closely associated with the nucleus, whereas in preparation to mitosis increases in size and defines the two opposite poles of the mitotic spindle to ensure an accurate chromosome segregation (Bettencourt-Dias and Glover, 2007; Mahen and Venkitaraman, 2012; Schatten, 2008).

Despite over 100 years passed since its discovery, only recently, its composition and structure started to be elucidated. The complementarity of different techniques such as RNA-mediated interference (RNAi), mass-spectrometry-based proteomics and centrosome isolation revealed a vast inventory of proteins, including as far as 500 proteins (Andersen et al., 2003), the majority harboring coiled-coil domains that might be permanently or temporarily associated with the human centrosome. Many of these proteins may not be involved in centrosome-specific functions and might only use centrosomes as a "docking station" to regulate cell-cycle specific events (Schatten, 2008). Numerous centrosome-specific proteins and respective orthologues have been identified and described in human and *Drosophila* centrosomes, including PCM-associated and regulatory proteins (Andersen et al., 2003; Dobbelaere et al., 2008; Goshima et al., 2007). Principal among PCM components, which have been studied in detail, are the conserved centrosomal proteins γ-tubulin, pericentrin and centrosomin (Cnn). Both are key players in

centrosome integrity, function and in the maturation process at the onset of mitosis characterized by an enrichment of numerous PCM components into this organelle which influences its MT-nucleation ability.

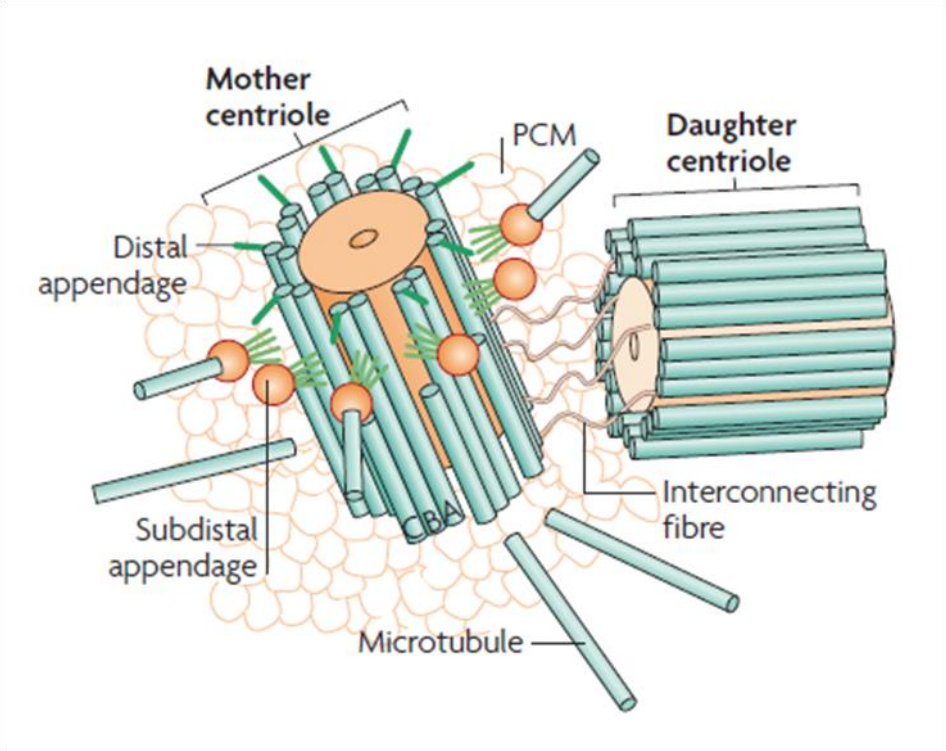


Figure 1. Centrosome structure. Schematic view of a typical animal centrosome, illustrating the mother and daughter centrioles that formed the centriole pair associated at each centrosome surrounded by a cloud of PCM proteins. Note that, specifically the mature “mother” centriole harbors distal and sub-distal appendages (adapted from Bettencourt-Dias and Glover, 2007).

γ-tubulin is a conserved eukaryotic protein known for its major role in MT nucleation and thereby vital for centrosome function. It is present in a ring-shape structure, the γ-tubulin ring complex (γ-TuRC) that promotes MT polymerization and organization (reviewed by Kollman et al., 2011). *Drosophila* centrosomin (or its mammalian orthologue CDK5RAP2) is an essential component for the recruitment of many PCM factors, such as γ-tubulin and pericentrin, and promotes the cohesion between centrioles and the PCM network (Buchman et al., 2010; Choi et al., 2010; Fong et al., 2008; Goshima et al., 2007; Lucas and Raff, 2007; Megraw et al., 1999; Vaizel-Ohayon and Schejter, 1999). Its depletion completely prevents centrosome maturation (Dobbelaere et al., 2008; Muller et al., 2010) and it was suggested that its Polo (PLK1 in

mammalian cells) dependent-phosphorylation during mitosis initiates centrosome maturation in flies (Dobbelaere et al., 2008). Pericentrin and AKAP450 family make part of a group of proteins harboring a centrosomal targeting (PACT) domain and known for their role in docking and recruiting regulatory components involved in MT nucleation (e.g. γ-TuRC) and in PCM integrity (Bettencourt-Dias and Glover, 2007; Lawo et al., 2012; Mennella et al., 2012; Schatten, 2008). *Drosophila* pericentrin-like protein (D-PLP) is the only PACT domain protein identified in flies. *d-plp Drosophila* mutants, although viable, are severely uncoordinated and the sperm is nonmotile, suggesting an additional role in cilia and flagella formation (Martinez-Campos et al., 2004).

Recently, the combination of 3D structured illumination microscopy (3D-SIM) and STORM (stochastic optical reconstruction microscopy) allowed a deeper look on centrosome architecture revealing a conserved high-order structure within PCM, as opposed to the traditional "amorphous" description. PCM organization is characterized by two overlapped layers: a radial and a concentric, in which proteins either are framed at specific sites in a layered fashion or extend outward from centriole wall. The PCM surface is composed by proteins in a toroidal arrangement linked to the outer wall of mother centriole such as SAS-4, followed by proteins extending out to the PCM periphery like SPD-2 (CEP192 in mammalian cells), Cnn and γ-tubulin. The radial distribution is strongly defined by D-PLP that binds its C-terminal PACT domain to the centriole wall and the N-terminal domain extends outwards in order to form a matrix of D-PLP extended fibrils that function as a scaffold for the recruitment of other PCM components. Interestingly, D-PLP fibrils formed an open gap (150-200 nm) on mother centrioles, coinciding with the position of daughter centriole assembly, which was not noticeable in the early stages of the cell cycle that precede centriole duplication (Fu and Glover, 2012; Lawo et al., 2012; Mennella et al., 2012; Sonnen et al., 2012).

Centrioles are at the foundation of centrosomes. Thus, an interaction between the centriole pair and the surrounding PCM was expected to exist. A symbiotic relationship between PCM and centrioles has been reported by several studies. Only the mother centriole within the centriole pair is able to anchor MTs through the sub-distal appendages (Piel et al., 2000) and centrioles define centrosome size by the incorporation of Cnn into the PCM, which seems to be driven by the components Asterless (Asl) and *Drosophila* Spd-2 (DSpd-2) (Conduit et al., 2010). Furthermore, the levels of centriolar protein SAS-4 were reported to set centrosome size (Kirkham et al., 2003) and SAS-4 itself was found in complexes with some centrosomal proteins, such as Asl, Cnn and D-PLP, suggesting a scaffolding role to tether PCM components within the centrosome (Gopalakrishnan et al., 2011). Also, the interaction SAS-4/Tubulin was shown to control PCM recruitment depending on the tubulin guanine-bound state (discussed in chapter 5) (Gopalakrishnan

et al., 2012). In agreement with an influence of centrioles in PCM assembly, the transiently centriole disintegration in HeLa cells upon the microinjection of an antibody directly against glutamylated tubulin (GT335) led to PCM dispersion, suggesting that the presence of centrioles allow the correct segregation and organization of the pericentriolar material (Bobinnec et al., 1998). Alongside, the PCM also exhibits an influence over centrioles. It was demonstrated that overexpression of pericentrin in S-phase arrested CHO cells induced the formation of large PCM clouds containing variable number of centrioles (Loncarek et al., 2008). Moreover, depletion of PCM proteins in the model organism *C. elegans* resulted in failure of centriole assembly in ~50% of the time, and the centrioles who were formed failed to reach full size (Dammermann et al., 2004). Considering the combined data, there is a strong support to the idea of a symbiotic model in which the interaction between centriole and PCM is important to assemble structural and functional normal centrosomes during cell division.

3.2. Overview of Centrosome Cycle

In active proliferating cells, the two functional centrosomes ensure accurate chromosome segregation through the formation of a bipolar mitotic spindle during mitosis. In order to be in harmony with cell cycle progression, the centrosome cycle must be highly coordinated with the DNA cycle. The Centrosome cycle depends on centriole duplication and behavior along the different phases of the cell cycle. The centrosome-centriole cycle can be divided into the following discrete and critical stages: (1) centriole disengagement; (2) centriole duplication and elongation; (3) centrosome maturation and (4) centrosome separation (Nigg, 2007) (Figure 3a). In interphase, a typical G_1 centrosome harbors two centrioles that are structurally and functionally different - a mother/mature centriole and a daughter or immature centriole assembled during the previous cell cycle. Centriole duplication starts in late G_1/early S transition with procentriole nucleation at right angle to the proximal end of each parental centriole. From S to mitosis, after the establishment of the basic centriolar structure, each procentriole starts to elongate reaching ~80% of its full-length during mitosis and eventually matures after a second cell cycle. A mature centriole differs from the younger one due to the acquisition of distal/sub-distal appendages and PCM enrichment. This final complete version of centriole has the ability to dock to the plasma membrane to promote ciliogenesis and to nucleate astral MTs to build the mitotic spindle. Thus, the assembly of a mature centriole able to promote and work as an independent and functional centrosome is a step-by-step process that needs two consecutive cell cycles (Avidor-Reiss and Gopalakrishnan, 2013; Azimzadeh and Marshall, 2010; Brito et al., 2012). The molecular mechanisms regulating centrosome-centriole cycle are for the most part poorly understood, nevertheless some experimental

works have already elucidated some essential regulators acting during the four stages of centrosome cycle.

During metaphase, each centrosome defining one of the two spindle poles contains a pair of tightly associated parental-progeny centrioles. In spite of the unknown nature of that linker (S-M linker) (Nigg and Stearns, 2011), it is known that upon exit of M phase (or early G_1 phase), the link between the two centrioles is lost in a process termed "centriole disengagement". Interestingly, it was shown in *Drosophila* that DSas-6-Ana2 complex might be involved in S-M linker functionality since their co-overexpression in spermatocytes formed short tubules (SAStubules) linking the inner region of the daughter centriole to the outer surface of the parental centriole, which is lost in meiosis I, coincident with centriole disengagement (Stevens et al., 2010b).

It is now imperative to approach the licensing model based on two distinct rules that centrosome cycle must obey in order to promote a correct centriole/centrosome number over successive cell divisions: (1) centrosomes duplicate once and only once in each cell cycle (cell cycle control) and (2) only one progeny centriole must arise next to each parental centriole (copy number control) (Nigg, 2007). Centriole disengagement has been proposed to be a key event for centriole licensing, rendering both centrioles competent to duplicate in the following S phase. Like chromosomes, centriole separation also requires the activity of separase, a protease responsible for driving sister chromatids separation prior to anaphase, as well as the kinase PLK1 (Tsou and Stearns, 2006; Tsou et al., 2009). Centriole disengagement as a license model for centriole duplication is supported by a series of experimental works. Wong and Stearns (2003) in an elegant cell fusion assay have shown that when G_2/S phase cells were fused, there was no centriole duplication, but when the fusion was between G_1/G_2-phase or G_1/S phase cells, the G_1 centrosomes replicate. Furthermore, Loncarek et al. (2008), by laser ablating the daughter centriole in S phase arrested cells, thereby artificially mimicking centriole disengagement, showed that resident mother centrioles were able to generate new daughter centrioles. Thus, both studies imply three major conclusions: first, centriole disengagement is a centriole-inherent permissive state to duplication; secondly, there is a centrosome-intrinsic-block to re-duplication which probably manifests itself in the already-duplicated and engaged centrioles (since G_2/S-phase fusions did not lead to centrosome duplication, in spite of permissive cytoplasmic conditions in S phase) and third, the centrosome somehow "senses" the presence of an immature centriole, blocking re-duplication events.

Coupled to DNA replication, the next stage in this sequence is centriole assembly in S phase in which each parental centriole "seeds" the growth of a new centriole (procentriole) which grows orthogonally in relation to the mother centriole. This process is controlled by a specific set of proteins discussed in detail in chapter 4. Nonetheless, the

cell cycle kinase CDK2 seems to be a critical factor to enhance centriole duplication (Hinchcliffe et al., 1999; Matsumoto et al., 1999; Meraldi et al., 1999). Formation of procentrioles coincides with an increase in CDK2 activity in S phase, and in complex either with cyclin A and E is essential to trigger centriole duplication through phosphorylation of nucleophosmin (NPM/B23) and Mps1 (Fisk and Winey, 2001; Okuda et al., 2000). However, the direct role of CDK2 in regulating centriole duplication is still controversial since it was shown that CDK2 activity is not essential for centriole duplication, but instead it seems to speed up procentriole formation (Duensing et al., 2006).

In the following cell cycle stages (from G_2 phase to mitosis) the two recently formed centrosomes must mature and separate in order to orchestrate the formation of a bipolar mitotic spindle. During late G_2 phase, centrosome separation seems to require the disintegration of the linker (G_1-G_2 tether) that mediates centrosome cohesion by linking the proximal ends of the two parental or previously disengaged centrioles (but not the two centrioles within a pair) (Nigg and Stearns, 2011). This linker is assembled right after centriole disengagement and remains temporarily associated with centrioles throughout interphase to ensure centrosome cohesion and to avoid premature centrosome splitting prior to mitosis.

Ultrastructural analysis revealed that rootletin, a conserved component of the ciliary rootlet, and C-Nap1 are key structural elements whose interaction is important for the functionality of this linker. Whereas C-Nap1 localization is restricted to the proximal ends of centrioles, rootletin forms fibers emanating from the proximal ends of both centrioles (Bahe et al., 2005; Mayor et al., 2000; Yang et al., 2006), suggesting that C-Nap1 functions as a "docking site" from where rootletin-based fibers attach and emanate. Upon entry into mitosis, this fibrous linker is disassembled through phosphorylation of C-Nap1 by the mitotic kinase Nek2 allowing the separation of the two independent centrosomes (each containing a pair of centrioles) and consequently mitotic spindle formation (Fry et al., 1998; Helps et al., 2000). Concomitantly with centrosome separation, the younger of the two parental centrioles (originated from the previous cell cycle) acquires distal and sub-distal appendages and enlarges its PCM with the recruitment of γ-TuRCs and other matrix components, thereby reaching full maturity and promoting centrosome maturation.

4. How to Build a Centriole ?

4.1. Centriole Architecture

Centrioles are microtubule-based, cylindrical and evolutionary conserved eukaryotic cell structures that exhibit a distinct nine-fold symmetric radial array of stabilized microtubules. They are polarized along the proximo-distal axis with the base commonly referred as the proximal end and its tips as distal ends. The typical size for a human mature centriole is approximately 200 x 500 nm, although these measures vary between organisms, as it is the case of *C. elegans* and *Drosophila* centrioles that tend to be shorter (Gonczy, 2012; Pelletier et al., 2006). Nonetheless, the signature of centriole architecture is undoubtedly the conservation of the 9-fold symmetry across evolution. Cryo-electron tomography studies have brought new and instructive data concerning centriole/basal body structure (Guichard et al., 2010; Guichard et al., 2013; Li et al., 2012). *How does the ninefold symmetry arise to build a highly ordered and complex structure as the centriole?.* In *Drosophila*, unicellular algae, many protozoa and vertebrates, the basic structure of centrioles relies on microtubule blades displayed into a 9-fold radial array (Loncarek and Khodjakov, 2009). This symmetry is established by the cartwheel, which is a structure located in the very proximal end of the centriole and composed of a central hub with 20-25 nm in diameter and ~100 nm in height from which nine spokes radiate outwards (Gonczy, 2012; Guichard et al., 2010) (Figure 2). Each stroke ends in a structure so-called Pinhead which bridges the central hub with centriolar microtubules. The cartwheel is the central piece on centriole organization and the first intermediate of centriole structure to appear during the early events of centriole biogenesis. Interestingly, despite its presence in immature centrioles, the cartwheel disappears from mature centrioles in some organisms (e.g. humans). In most species, including human centrioles, nine sets of microtubule triplets decorate the outer surface of the centriole and are linked to the cartwheel through the stroke pinheads. From the inside out, each triplet comprises an A-microtubule, B-microtubule and a C-microtubule, in which only the A-microtubule is complete being formed by 13 protofilaments. The A-microtubule is oriented toward the center of the centriole and is connected to the pinhead of each stroke, B-microtubules attach to A-microtubules and consequently C-microtubules associate with neighboring B-microtubules. Moreover, A-microtubules can bind to C-microtubules from the previous triplet forming an A-C linker. This microtubule triplet pattern is only characteristic of the proximal-end of centrioles since the distal-end displays double microtubules (A- and B-microtubules) (Guichard et al., 2013; Li et al., 2012).

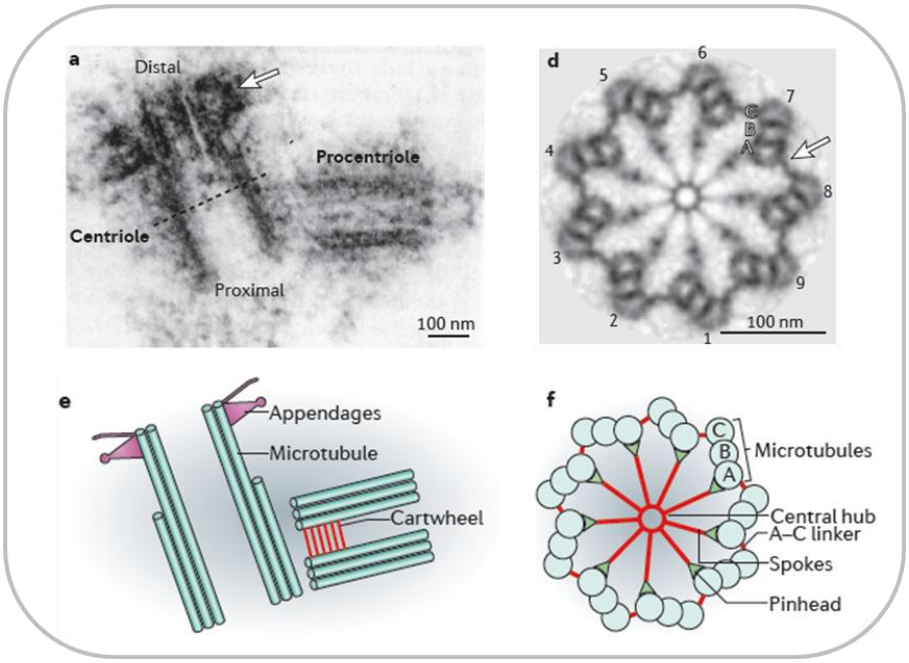


Figure 2. **Centriole and Cartwheel architecture. (a,d)** The ultrastructure of a resin-embedded centriole and the cartwheel purified from human cells and *Chlamydomonas reinhardtii*. Part **(a)** shows the side view of a mature human centriole. The proximal and distal ends of the centriole are indicated. The arrow points to distal and subdistal appendages present on the sides of the distal part of the centriole. Part **(d)** shows a cross-section of the proximal part of a *C. reinhardtii* centriole. Note the central tube from which nine spokes emanate that radiate towards the vicinity of triplet microtubules. The A-,B- and C-microtubule are indicated and the arrow points to the A-C linker. **(e)** Schematic representation of a centriole and procentriole pair in a human cell. **(f)** Schematic representation of the cartwheel viewed from the proximal end (adapted figure from Pierre Gönczy, 2012).

Interestingly, it was recently demonstrated that the pinhead structure plus the A-C linker are polarized structures that might be responsible for dictating the directionality of centriolar microtubule assembly, as well as the chirality of microtubule triplets (Guichard et al., 2013). An amazing exception to this cartwheel-based centriole structure is represented by *C. elegans* centrioles. Electron microscopy studies revealed that they do not comprise the cartwheel structure and, instead, follow a more simple architecture formed by a central tube on which nine sets of centriolar microtubules are directly attached (Pelletier et al., 2006). Additionally, the microtubule number can also vary between species. For example, *C. elegans* centrioles are made only by singlet

microtubules whereas *Drosophila* centrioles in embryos and most tissues are formed by doublet microtubules, and specifically in the spermatocytes of male germ line, centrioles do exhibit centriolar triplets of microtubules (Carvalho-Santos et al., 2010; Debec et al., 2010). In spite of these variations on centriole structure, the nine-fold symmetry is always maintained.

A typical interphase G_1 cell harbors one centrosome built from a pair of centrioles linked by a flexible connection. Through the following cell cycle stages, a series of events take place including centriole replication, elongation and maturation. A cryo-electron tomography analysis of centrosomes isolated from human lymphoblasts by Guichard and co-workers have revealed new insights about the initial structural events involved in centriole duplication. In the nascent procentriole, centriolar singlets A-microtubules were observed which seemed to be capped by a conical structure at their proximal or minus end resembling the γ-tubulin ring complex (γ-TuRC). This suggests, in association with other studies (Dammermann et al., 2008; Dammermann et al., 2004; Kleylein-Sohn et al., 2007), the involvement of γ-TuRC in nucleating each A-microtubule, which grows unidirectionally from the proximal to the distal (plus) end during centriole assembly. Accordingly, the distal end of A-microtubule is not closed and, instead, it showed outward curved extensions characteristic of growing microtubules. In contrast with the minus end of A-microtubule, B- and C- microtubules showed always open and outward curved extensions at both proximal and distal ends. Therefore, nucleation of these last microtubules (B and C) is not mediated by γ-TuRC, but it follows a template-dependent mechanism, in which A- and B-microtubules are the templates for a bidirectionally growth (from their plus and minus ends) of the B- and C-microtubules. Interestingly, the attachment of A-, B- and C-microtubules for the centriole wall formation occurs independently and without any specific order or position. As B- and C-microtubules reach the minus end of A-microtubules, their proximal ends become blunt and just the distal-ends continues to grow until completion of the microtubule triplet blades (Guichard et al., 2010). In a mature centriole, A-microtubules lose the closest conformation of the proximal end indicating that γ-TuRC is no longer necessary, being removed when the centriolar microtubule wall is fully developed. The composition of B- and C-microtubules is still unknown, but it seems that ε- and δ-tubulin are potential candidates to take in consideration, since their mutation in *Chlamydomonas* and *Paramecium* disrupts the MT triplet arrangement. Nevertheless these two tubulin isoforms are absent from *D. melanogaster* proteome suggesting an alternative mechanism for B- and C-microtubules nucleation (reviewed in Azimzadeh and Marshall (2010), Brito et al. (2012) and Gonczy et al. (2012)). The answer to this question awaits further experiments.

4.2. Centriole-assembly pathway: action of a core ancestral protein module

Centrioles are structurally complex organelles, but surprisingly the mechanism orchestrating centriole assembly relies on a few and evolutionary conserved core of proteins. A long-standing question in centriole biology was focused on the players and the way they interacted to initiate and produce a centriole. The first cues concerning the molecular mechanism involved in centriole biogenesis came from studies in *C. elegans* embryos. Genetic analysis and RNA_i-based screens revealed a hierarchical molecular cascade in which only five proteins are specifically required for centriole duplication: the coiled-coil proteins SPD-2, SAS-4, SAS-5, SAS-6 and the kinase ZYG-1 (Dammermann et al., 2004; Delattre et al., 2004; Kemp et al., 2004; Kirkham et al., 2003; Leidel and Gonczy, 2003; O'Connell et al., 2001). During centriole assembly, these proteins are sequentially recruited to centrioles (Delattre et al., 2006; Pelletier et al., 2006), where procentriole nucleation is triggered by the PCM protein SPD-2 that recruits ZYG-1 to the procentriole. The protein kinase ZYG-1 is responsible for the recruitment of the structural proteins SAS-5 and SAS-6, who physically interact (Leidel et al., 2005) to build the central tube and to recruit SAS-4, which induces the assembly of nine singlet microtubules to the outer wall of the emerging centriole. However, given that *C. elegans* centriole architecture is atypical and divergent compared with *Drosophila* and mammalian centrioles (Pelletier et al., 2006), it became imperative to address if the same protein module governing centriole duplication in worms was transversal to other organisms.

An elegant study conducted by Kleylein-Sohn *et al.* (2007) unveiled how centriole biogenesis is governed in human cells. Taking advantage of centriole induction by PLK4 overexpression (Habedanck et al., 2005; Rodrigues-Martins et al., 2007b) in association with siRNA mediated depletion of individual centriolar proteins, a similar set of proteins associated with *C. elegans* centriole assembly were described to control this process in humans cells. Five proteins were identified to be essential for centriole duplication, specifically human SAS-6 (hSAS-6), CPAP (functional homologue of SAS-4/DSas-4 in *C. elegans* and *Drosophila*), CEP135, CP110 and γ-tubulin. A putative sequential centriole assembly pathway was described in which Polo-like kinase 4 (PLK4) is an upstream and key regulator essential to trigger procentriole assembly, and does not depend on the presence of any of the other proteins to localize to centrioles (Bettencourt-Dias et al., 2005; Habedanck et al., 2005; Kleylein-Sohn et al., 2007). Although PLK4 is not an homologue of *C. elegans* ZYG-1, it seems that both proteins work in an analogous way to initiate centriole duplication in different organisms. Thus, in human cells, PLK4 activation on the surface of the parental centriole triggers procentriole assembly and is crucial for the recruitment of hSAS-6 and STIL (functional homologue of *C. elegans* SAS-5 or *Drosophila* Ana2) (Vulprecht et al., 2012), which in turn are needed for CPAP loading in the

procentriole (reviewed by Gonczy, 2012). CPAP was shown to be a substrate for PLK2 and its phosphorylation (CPAP residues 589-595) is critical for procentriole assembly as well as for its stabilization in the nascent centriole (Chang et al., 2010).

Moreover, in agreement with Kleylein-Sohn et al. (2007), in which CEP135 was identified as a core component of centriole duplication, it was recently shown that CEP135 directly interacts with hSAS-6 and CPAP, indicating that these three proteins might regulate centriole biogenesis as a complex. It was also identified in its protein structure a MT-binding domain, elucidating a potential role for CEP135 in mediating CPAP-dependent centriolar microtubule assembly (Lin et al., 2013). Furthermore, it was shown that STIL N-terminal domain interacts and eventually recruits CPAP to the procentriole (Cottee et al., 2013; Vulprecht et al., 2012). Strikingly, as opposed to *C. elegans* and *Drosophila*, human STIL does not seem to form a stable complex with hSAS-6. Nevertheless, the siRNA-mediated depletion of STIL significantly decreased hSAS-6 levels at centrosome (Vulprecht et al., 2012), and STIL centriolar localization was also affected upon hSAS-6 depletion (Arquint et al., 2012), suggesting that STIL and SAS-6 might be partially interdependent for their localization at centrioles (Vulprecht et al., 2012).

SPD-2 ortholog in humans, called CEP192, seems also to be required for centriole duplication in human cells (Zhu et al., 2008). CEP192 was recently found to cooperate with CEP152 for the centriolar recruitment of PLK4 during centriole duplication, indicating an important and direct role of this PCM component in human centriole biogenesis (Kim et al., 2013; Sonnen et al., 2013).

As expected, the sequential model proposed in mammalian cells extends to *D. melanogaster*. In a genome-wide screen to dissect centriole duplication and centrosome maturation in *Drosophila* cells (S2R+), from among 119 centrosomal-related genes that were analyzed, only nine genes were identified to be directly involved in centriole assembly. From those nine genes, three coded for the well-known SAS-4, SAS-6 and SAK (*Drosophila* homologue of PLK4) proteins and another three genes coded for a set of proteins so-called Ana1, 2 and 3 (previously identified by Goshima et al., 2007). As emphasized previously, SAK (or PLK4 in human cells) is a master regulator in centriole assembly, whose depletion in *Drosophila* impairs centriole duplication and flagella formation (Bettencourt-Dias et al., 2005). Overexpression of Ana1 and Ana2 formed extra centrioles suggesting a potential role in centriole duplication in *Drosophila* cells (Dobbelaere et al., 2008). Latter, Ana2 was identified either as a conserved centriole duplication factor and as orthologue of STIL/SAS-5 based on weak sequence similarities (Stevens et al., 2010a), as well as Ana3 was described to be important for centriole structural integrity and cohesion, but not for centriole duplication. On the other hand, the precise function of Ana1 on centriole assembly is still not known. However, it has been

shown that its RNAi depletion in S2 *Drosophila* cells led to a significant decrease in SAS-6 levels at centrosome, indicative of an important role in centriole duplication (Goshima et al., 2007).

As opposed to mammalians and worms, SPD-2 is not required for centriole assembly in *Drosophila* (Dix and Raff, 2007), therefore flies and humans have one additional duplication factor, Asterless (Asl; human orthologue CEP152) (Blachon et al., 2008; Dobbelaere et al., 2008; Dzhindzhev et al., 2010). This coiled-coil protein was shown to be extremely important for centriole duplication in *Drosophila* and human cells since: (1) directly binds to SAK/PLK4's cryptic polo box domain (CPB, conserved motif in PLK4 orthologs) mediating its centrosomal localization in *Drosophila* and human cells; (2) interacts through N-terminal domain of CPAP or *Drosophila* Sas-4 (DSas-4), recruiting human CPAP to centrosomes and (3) its overexpression led to supernumerary centrosomes in *Drosophila* and human cells. Thus, it was proposed that Asl/CEP152 acts upstream of PLK4 during centriole biogenesis, acting as a scaffold protein for both PLK4 and SAS-4/CPAP and triggering procentriole nucleation in flies and humans (Cizmecioglu et al., 2010; Dzhindzhev et al., 2010).

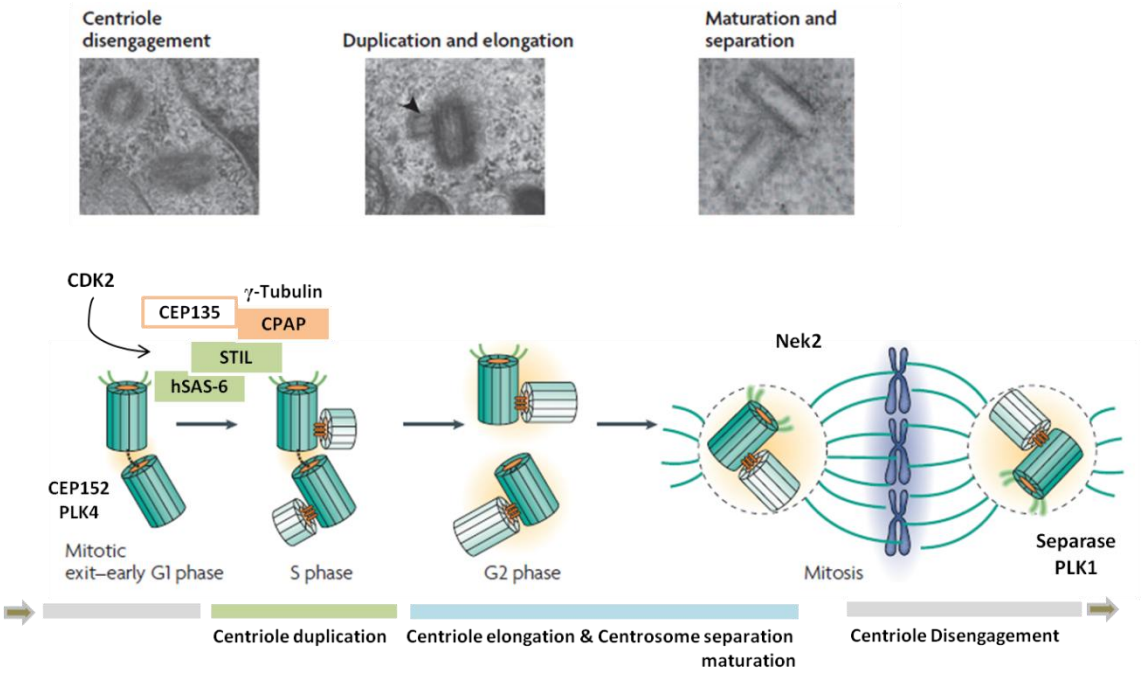
As in the other model organisms, *Drosophila* SAS-6 (DSas-6) and Ana2 cooperation is crucial for centriole assembly (Stevens et al., 2010a). DSas-6 depletion in *Drosophila* spermatocytes and S2 cells led to a reduction in centriole number, and the few proportion of formed centrioles were smaller and lost the typical 9-fold symmetry. In opposition, DSas-6 overexpression in *Drosophila* embryos and unfertilized eggs resulted in *de novo* formation of MTOCs, in which were detected irregular tube-like structures that resembled incomplete centrioles with wrong orientation and symmetry (Rodrigues-Martins et al., 2007a). Moreover, only when DSas-6 was overexpressed in combination with its binding partner Ana2 in *Drosophila* spermatocytes, there was the assembly of ordered tubule-like structures (SAStubules) similar to the inner centriole cartwheel. Strikingly, in contrast to what was previously reported about DSas-6 overexpression in *Drosophila* eggs and embryos (Rodrigues-Martins et al., 2007a), SAStubules were not formed when DSas-6 and Ana2 were overexpressed individually or under combined DSas-4 / Asl overexpression in *Drosophila* spermatocytes, indicating that DSas-6 and Ana2 are indispensable players and do need to cooperate to drive the formation of the cartwheel in *Drosophila* centrioles (Stevens et al., 2010b).

Besides the reported interaction between DSas-6/Ana2, DSas-4 is also a well-known interacting protein. Its depletion led to centriole loss in *Drosophila* flies and impaired centriole duplication in *C. elegans* embryos and human cells (Basto et al., 2006; Kirkham et al., 2003; Leidel and Gonczy, 2003; Tang et al., 2009). Recent structural analysis identified the TCP region as a conserved binding domain of CPAP/DSas-4/SAS-4

that interacts with a short (~40 aa) proline-rich region at N- terminus of STIL/Ana2, which was not retained in *C. elegans* SAS-5. This interaction allows the formation of DSas-4/Ana2 or CPAP/STIL complex during centriole duplication (Cottee et al., 2013; Vulprecht et al., 2012). Furthermore, it should be noted that since PLK2 is not conserved in invertebrates (Chang et al., 2010), an eventual DSas-4 phosphorylation by PLK2 during centriole assembly is unlikely to occur in *Drosophila,* as it happens in human cells, suggesting that other protein kinases regulate and activate DSas-4 during the early events of centriole assembly in flies. Since Asl is a scaffold protein for both DSas-4 and PLK4, it is conceivable that Asl might serve as a bridge for a potential interaction and phosphorylation of DSas-4 by PLK4 (Chang et al., 2010; Dzhindzhev et al., 2010). Interestingly, it was shown that the stably incorporation of *C. elegans* SAS-4 into the new centriole requires γ-tubulin, which is believed to contribute for centriolar microtubule nucleation (Dammermann et al., 2008; Kleylein-Sohn et al., 2007). This γ-tubulin-dependent stabilization into the daughter centriole is likely to happen in humans and *Drosophila* cells since it was shown that CPAP and DSas-4 co-immunoprecipitate with γ-tubulin (Gopalakrishnan et al., 2011; Hung et al., 2000).

In summary, procentriole nucleation in dividing *Drosophila* and human cells occurs at late G_1/early S transition and is triggered by Asterless that recruits PLK4 to the site of procentriole assembly. PLK4 or SAK presence and activation on the surface of the parental centriole triggers the recruitment of the first structural proteins to the nascent procentriole: DSas-6/hSas-6, Ana2/STIL and CEP135. DSas-6 and Ana2 act as a complex to start cartwheel assembly orthogonally to the pre-existing centriole and to establish the nine-fold symmetry of the new centriole. Two landmark crystallography studies (Kitagawa et al., 2011b; van Breugel et al., 2011) revealed that SAS-6 self-assemble into oligomers through its N-terminal globular head domain. First, SAS-6 monomers initially form dimers through a coiled-coil domain and the resulting dimers can then associate via the head-group interaction into a ring-like conformation to form a nine-fold symmetrical central-cartwheel structure with the coil-coiled domains forming spokes radiating from a central hub (reviewed by Cottee et al., 2011). CEP135 may act as a bridge between the cartwheel and centriolar microtubules by forming the pinheads, as well as be involved in centriolar MT stabilization. Following cartwheel assembly, centriolar microtubules are attached and stabilized onto the periphery of the cartwheel, promoting daughter centriole elongation in G_2/mitosis. This task is performed by DSas-4/CPAP and γ-tubulin (Figure 3). The distal end of procentriole is capped by CP110, which promotes the stabilization of nascent procentriole and controls centriole elongation.

a



b

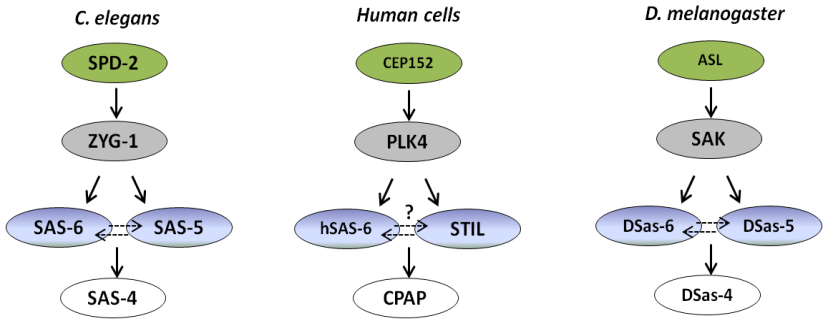


Figure 3. (a) The Centrosome cycle. Electron microscopy (TEM) images of centrioles in the respective cell cycle stage. Schematic illustration of the four major events of the centrosome cycle (centriole disengagement, centriole duplication and elongation, centrosome maturation and separation). Mature centrioles are depicted with schematic green appendages at their distal ends. Procentrioles are shown in light green. The key protein machinery involved in the human centriole-centrosome duplication-segregation cycle is shown (adapted from Bettencourt-Dias and Glover, 2007) **(b) Evolutionary conserved module for centriole assembly.** Core components of centriole duplication pathway in *C. elegans*, human cells and *D. melanogaster.* Arrows indicate the hierarchy in the recruitment. Dashed arrows indicate the physical interaction between SAS-6/DSas-6 and SAS-5/Ana2. Note that in human cells, this physically interaction was still not verified.

5. SAS-4: a multi-faceted protein

Apart from SAS-4 requirement for centriole biogenesis, this protein plays a variety of other roles linked with centrosome biology. The scientific history of SAS-4 starts in the year 2000 with the discovery of its related human protein, CPAP (centrosomal P4.1-associated protein) associated with the γ-tubulin complex (Hung et al., 2000). Curiously, the main goal of the previous study was not completely focused on the centrosome, but rather to investigate the possible functions and binding partners of one of the multiple nonerythroid protein 4.1 isoforms (4.1R-135) in mitotic spindle pole organization. Consequently, it was identified an interaction of 4.1R-135 with a novel centrosomal protein called CPAP (also known as CENPJ) and, although the authors successfully demonstrated the specific association between those two proteins, it was not possible to conclude the functional role of the 4.1R-135/CPAP association. Nevertheless, the novelty of this study lies on the discovery of the tight association of CPAP with the γ-tubulin complex and its localization within the center of microtubule asters along the cell cycle.

CPAP is a coiled-coil protein structurally characterized by five α- helical coiled-coil segments followed by a conserved repeated sequence of glycines on its carboxyl terminus, termed G-box domain. Additionally, there are four potential phosphorylation sites and the fifth coiled-coil segment plus the G-box domain exhibits 76.6% homology with human TCP10 (t-complex responder gene) (Cottee et al., 2013; Hung et al., 2000).

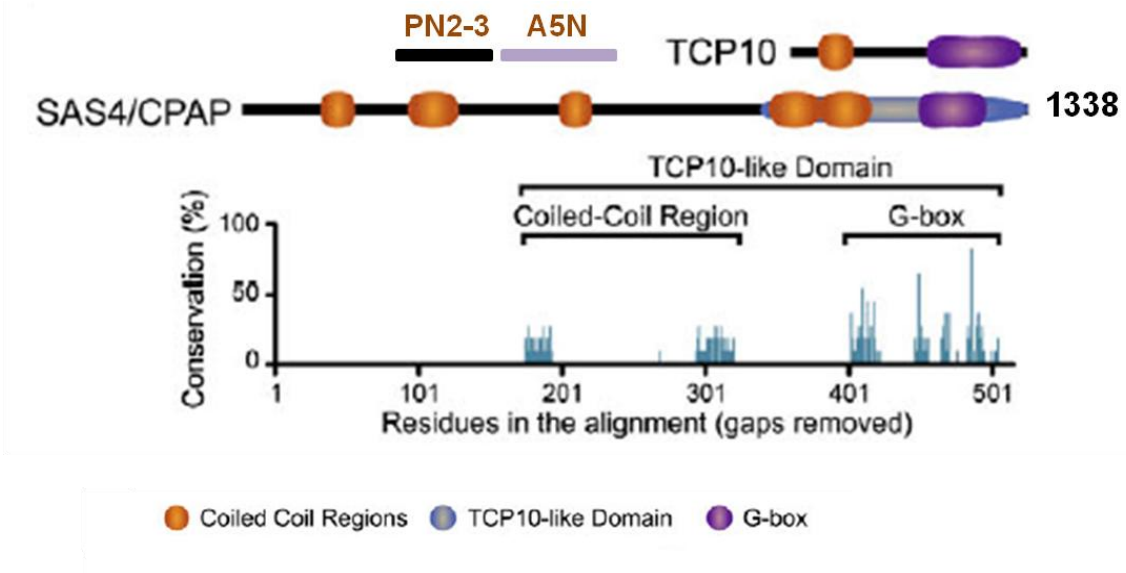


Figure 4. Molecular Architecture of human CPAP. Schematic representation of SAS-4/CPAP indicating the five predicted coiled-coil domains, the G-box region (residues 1159-1264), the PN2-3 (residues 311-422) and the A5N (residues 423-607) domains. The

G-box region is located at C-terminus being integrated in the TCP10 domain, and is characterized by several motifs of glycine or glutamine. The sequence conservation plot of the different SAS-4/CPAP orthologues is shown below (adapted from Carvalho-Santos et al. 2010). Note that in addition to the TCP10-like domain, the PN2-3 and A5N regions are also conserved among humans, *Danio rerio*, *D. melanogaster* and *C. elegans* (Hatzopoulos et al., 2013).

In addition to what was previously described about CPAP structure, it was found a specific and unique motif on its N-terminal domain localizing to the plus-ends of newly assembled MTs, the PN2-3 polypeptide (amino acid residues 311-422), that seems to be responsible for MT disassembly as well as to associate with tubulin dimers (Hung et al., 2004). Interestingly, this domain has no homology with other MT depolymerizing proteins suggesting a distinct molecular mechanism for MT disassembly.

Cormier and co-workers took advantage of the previous work and unveiled the molecular interaction between tubulin/PN2-3 domain. Apparently, PN2-3 region of CPAP interferes with β-tubulin longitudinal associations preventing MT assembly by sequestering non-polymerized tubulin to form a binary 1:1 complex (Cormier et al., 2009). Surprisingly, in complement to CPAP MT-destabilizing capacity, it was found a MT-binding domain (A5N polypeptide, residues 423-607) located just beyond PN2-3 region that is able to directly bind to polymerized MTs and promote microtubule stability (Figure 4). These dynamic MT properties were also found to be shared by DSas-4, which present a conserved motif (~20-aa) within the PN2-3 region and, like human CPAP, possesses the ability to depolymerize MTs (Hsu et al., 2008). The molecular mechanism governing the transition between MT-binding or tubulin sequestering by CPAP is still to be elucidated. Additionally, it seems that the coiled-coil four domain (CC4) is the main responsible for mediating CPAP centriolar localization (Kitagawa et al., 2011a). The PN2-3, A5N and G-box domains are the most conserved regions between human CPAP and its orthologues in *D. melanogaster* and *C. elegans* (Carvalho-Santos et al., 2010; Hatzopoulos et al., 2013; Kitagawa et al., 2011a) (Figure 4).

The first cues on the connection between SAS-4 and centrioles came out in two important studies (Kirkham et al., 2003; Leidel and Gonczy, 2003) describing not only the relationship between SAS-4 and centriole duplication, but also its influence on the surrounding PCM. SAS-4 is conserved in all organisms bearing centrioles (Carvalho-Santos et al., 2010; Hodges et al., 2010) and localizes to the wall of the centriole as well as to the PCM (Dammermann et al., 2008; Fu and Glover, 2012; Gopalakrishnan et al., 2011; Kirkham et al., 2003; Kitagawa et al., 2011a; Kleylein-Sohn et al., 2007; Leidel and

Gonczy, 2003; Sonnen et al., 2012). SAS-4 is present throughout the cell cycle in *C. elegans* centrioles (Kirkham et al., 2003) and it was recently shown that human CPAP persists until late mitosis being targeted for degradation by the APC/C-Cdh1 complex (Tang et al., 2009). Moreover, FRAP (fluorescence recovery after photobleaching) analysis in *C. elegans* embryos revealed the dynamics of SAS-4 during the cell cycle showing that it is recruited to the site of newly centriole assembly and reached its maximum levels on S phase, but it remains in dynamic equilibrium with cytoplasmic pool until late prophase. Coincident with the addition of microtubules to the centriole wall during late prophase, centriolar SAS-4 is stabilized in a process that depends on γ-tubulin and microtubule assembly (Dammermann et al., 2008). Strikingly, in contrast to *C. elegans* SAS-4, it was shown that human CPAP exchanges throughout the cell cycle with a cytoplasmic pool and it is not stabilized after its incorporation into centrioles. In this respect, it will be interesting to address the dynamics and regulation of *Drosophila* SAS-4 along the cell cycle.

In opposition to the deregulation of centriole number originated by overexpression of Asl/CEP152, PLK4/SAK, SAS-6 and SAS-5/Aan2/STIL (Arquint et al., 2012; Dzhindzhev et al., 2010; Habedanck et al., 2005; Kleylein-Sohn et al., 2007; Peel et al., 2007; Rodrigues-Martins et al., 2007b; Strnad et al., 2007; Vulprecht et al., 2012), CPAP overexpression generates abnormally long centrioles. In fact, it appears that procentriole elongation depends mainly on the synergy between the two centriolar proteins CPAP and CP110. Tang et al. (2009) showed that exogenous CPAP overexpression induces elongated filaments (PLSs) that resemble centriolar structures with longer MTs. Surprisingly, those filaments comprise some characteristics that resemble elongated procentrioles: (1) are formed after G_1 phase with a rapid growth between G_2-M phases, (2) are positive for acetylated tubulin and (3) show normal incorporation and localization of centriolar proteins, like hSAS-6 and CP110. Besides, mutation of the CPAP-specific tubulin binding domain affected the formation of PLSs representing a very important link for the CPAP-mediated MT assembly during centriole elongation (Tang et al., 2009). Similar results of CPAP´s role in centriole length were also reported in two independent studies conducted by Kohlmaier et al. (2009) and Schmidt et al. (2009). In contrast, excess of CP110 has exactly the opposite scenario, in which its depletion resulted in microtubule extensions similar to the elongated centrioles from CPAP overexpression. These microtubule extensions stained positive for polyglutamylated tubulin and to a variety of centriolar proteins, but not for cilia specific markers (e.g. IFT88) (Schmidt et al., 2009). Conversely, CP110 overexpression in synchrony with CPAP depletion resulted in inhibition of abnormally large centrioles (Schmidt et al., 2009), demonstrating that both proteins work together in an antagonist way to control centriole size.

Accordingly with its PCM localization, the amount of SAS-4 was also shown to dictate centrosome size. Partial depletions of this protein (by varying the time between injection of SAS-4 dsRNA into hermaphrodites worms) led to incomplete centrosomes with less PCM and defects in MT nucleation capacity in *C. elegans* embryos (Kirkham et al., 2003). However, the last study did not clarify if the asymmetry in centrosome size was accomplished through a direct regulation of SAS-4 or by an indirect action through centriole formation.

Gopalakrishnan and co-workers worked on the previous issue and in two recent papers (Gopalakrishnan et al., 2012; Gopalakrishnan et al., 2011) they were able to demystify the influence of SAS-4 on centrosome maturation. First, it was shown that SAS-4 scaffolds cytoplasmic complexes of centrosomal proteins, including Cnn, D-PLP, β-tubulin and Asl (S-CAP complexes) via its PN2-3 domain, and tethers them to a procentriole through its C-terminal domain allowing the establishment of a mature centrosome along the rest of the cell cycle. Using a cell free system in which "stripped centrosomes"[2] were created with high salt concentrations, it was demonstrated that in the presence of a recombinant SAS-4 protein, Asl and Cnn were able to bind to centrosomes stripped of PCM. In opposition, when SAS-4 was removed from the mixture leaving only Asl and Cnn, both proteins were not able to bind alone (Gopalakrishnan et al., 2011).

In a second study, they reported and clarified the molecular mechanism by which SAS-4 regulates and tethers the S-CAP complexes into the centrosome. As the association between SAS-4 and tubulin was the first interaction to be reported (Gopalakrishnan et al., 2011; Hung et al., 2004; Hung et al., 2000), they analyzed how far this interaction could influence PCM recruitment. By combining several biochemical and genetic tools with a deep knowledge about the biochemical properties of the tubulin molecule, it was developed an unprecedented model on the SAS-4/tubulin interaction based on the activity of SAS-4 as a tubulin GAP[3] to control the GTP/GDP-bound state of tubulin. Briefly, it was shown that cytoplasmic free tubulin-GTP binds to SAS-4 and, since SAS-4 has GTPase activity, it is able to hydrolyze GTP into GDP. The tubulin-GTP/GDP inter-conversion becomes the molecular switch that regulates the SAS-4-dependent formation of the centrosomal complexes. SAS-4/tubulin-GDP positively promotes the assembly of stable S-CAP in the cytoplasm, whereas the association with tubulin-GTP negatively regulates the interaction between SAS-4 and centrosomal proteins (e.g. Cnn).

It is also possible that MT depolymerization can contribute and enhance the formation of S-CAP complexes by releasing tubulin-GDP to the cytoplasm, which directly associates with SAS-4. After being tethered to the centrosome through the C-terminal

[2] PCM components were removed. It is composed of centrioles and a salt-stable centrosome matrix.
[3] GTPase- activating proteins.

region of SAS-4, the tubulin GTP/GDP-bound status is again changed by the centrosome with the conversion of GDP into GTP, promoting the disassembly and release of SAS-4 and tubulin to the cytoplasm. Interestingly, the interaction SAS-4/tubulin controls either centrosome function and centriole length (Kitagawa et al., 2011a; Schmidt et al., 2009; Tang et al., 2009), but it seems not to have an influence on centrosome number.

A potential role for CPAP in centrosome splitting was also described based on its self-interaction through the fifth coiled-coil domain (CC5) to form homodimers. In fact, the transition between monomeric and dimeric CPAP seems to be fundamental for a faithful cell division. In interphase, CPAP homodimerization seems to be essential to negatively regulate centrosome splitting. As cells progress through G_2/M phases, centrosomes separate in order to build a bipolar spindle, and the homodimerization of CPAP is abolished by phosphorylation, blocking its self-interaction and promoting centrosome separation. Although it was not identified the kinase responsible for CPAP phosphorylation, neither the molecular mechanism by which centrosome splitting is inhibited, this study shows that CPAP dynamics during the cell cycle must be controlled to guarantee an accurate cell division (Zhao et al., 2010).

Deregulations in centriole/centrosome cycle are linked to several human diseases including cancer, obesity and polycystic kidney disease (Nigg and Raff, 2009). Human neurodevelopmental disorder (autosomal recessive primary microcephaly, MCPH) is characterized by small brain size associated with mental retardation, and it has been linked with mutations in several loci coding for centrosomal proteins. Three specific mutations in locus MCPH6, encoding a modified version of CPAP, were shown to be associated with MCPH disorder (Bond et al., 2005). Specifically, two of those mutations encompass a missense mutation (E1235V) within the TCP domain and premature stop codons that result in a protein lacking the TCP domain. By combining siRNA of endogenous CPAP with the expression of GFP-fusions of CPAP-MCPH mutations in human cells (U2OS and HeLa), Kitagawa et al. (2011) demonstrated that impaired centriole biogenesis derived from CPAP mutations led to an increase in asymmetrical spindles associated with incomplete centrosomes presenting few astral MTs. Consequently, the asymmetry of the mitotic spindle led to a randomization and defects in spindle positioning. Since the correct spindle positioning during the first divisions of neuroepithelial progenitor cells is critical for a proper brain development, MCPH mutations, by affecting centriole assembly promote an incorrect spindle positioning orientation and consequently failure to produce the correct number of neurons during brain development (Kitagawa et al., 2011a).

6. Centriole Biogenesis: Different origins, one Goal

Redundancy has always made part of nature. The discovery that genetic code has multiple codons which code for the same amino acid, allowed us to wonder until what extent this "feature" is used for other processes by the cell. Previously it was described how centrioles are inherited during cell division, in which the assembly of the new centriole during the transition G_1/S occurs on a pre-existing centriole that serves as a template. This last centriole assembly mode is called canonical or template mechanism and suggests that the mother centriole somehow acts as a template providing a site or pattern for the assembly of the new centriole. *But is the template pathway unique and exclusive for centriole assembly and inheritance?*.

In fact, this template mechanism has been challenged by peculiar situations, either naturally or experimentally, in which centrioles arise without a pre-existing centriole, or in another words, centrioles are formed *de novo*. While the templated/canonical pathway is the most common pathway for centriole propagation in somatic cells, the *de novo* mode of centriole assembly is frequently associated with the development of many organisms, including parthenogenetic species (e.g. *Nasonia vitripenins*, sea urchin, *Spisula*), the ameboflagellate *Naegleria*, gametogenesis in lower plants and fungi and, even in embryogenesis (reviewed in Debec et al., 2010, Marshall et al., 2001). Probably, the most intriguing and surprising case of *de novo* centriole formation in higher animals was reported during the early events of mouse development. As it was already described, a common feature during oogenesis is centriole loss (e.g. *D. melanogaster*). Nevertheless, there are cases (e.g. mouse) in which both parental and maternal centrioles are eliminated during gametogenesis. Thus, after fertilization, the very first embryonic divisions of the mouse zygote are acentrosomal, and only during blastomere stage of development (16 to 32 cells) centrioles assemble by the *de novo* pathway in a proper number. Interestingly, upon the first round of centriole assembly, those *de novo* formed centrioles duplicate and segregate by the canonical pathway thereafter (Szollosi et al., 1972). Another surprising case of centriole formation happens in differentiated ciliated epithelial cells. In mammals, multiciliated epithelium is found in the airways, the oviduct and the ventricular system of the brain. In order to give rise to hundreds of centrioles (that will ultimately be converted in cilia) *per* cell, ciliated epithelial cells developed a template-free mechanism for centriole assembly based on the generation of hundreds of centrioles around a non-microtubule based/non-centriolar structure called deuterosome (Dawe et al., 2007; Vladar and Stearns, 2007).

Although the first reports of *de novo* centriole formation were related to specific events as embryonic development or in differentiated/specialized cells, a series of experiments have proved that somatic cycling cells can also drive centriole biogenesis by

the *de novo* pathway. It is worth noting three studies (Khodjakov et al., 2002; La Terra et al., 2005; Uetake et al., 2007) conducted during the last decade in which the *de novo* centriole assembly was induced in cycling vertebrate somatic cells in culture, which would normally depend on a templated centriole production. Taking advantage of laser microsurgery to ablate the whole centrosome or only the mother centriole, both studies demonstrated that centrioles arise *de novo* in cultured transformed and non-transformed mammalian cells (e.g. CHO, HMEC, RPE1 and HeLa), thereby elucidating important features of this process.

First, *de novo* centriole formation in S phase arrested cells is preceded by extensive clouds of typical electron-dense material without centrioles formed after 8-10h upon centrosome ablation; second, centrioles only appear approximately 24h after ablating the centrosome; third, *de novo* centrioles are able to mature, nucleate microtubules and replicate after completion of a second cell-cycle; fourth, the presence of a single centriole is sufficient to inhibit *de novo* centriole pathway. At last, centriole assembly is restricted to S phase, since G_1-arrested cells for at least 72h did not form *de novo* centrioles as well as after centriole assembly in S phase there was not an increase in centriole number (centrin-GFP dots) throughout the cell cycle. Interestingly, in all cases of *de novo* centriole assembly, there was the formation of multiple centrioles (~2-14 per cell) exhibiting morphological intermediate stages, ranging from electron dense amorphous clouds with only two or three microtubule blades, open centriolar cylinders until more morphological normal centrioles. In addition to the laser ablation studies, the *de novo* centriole formation was also demonstrated *in vivo* in the unicellular green algae *Chlamydomonas*, in which *vfl2* mutants that did not inherit centrioles due to a mutation on the centrin gene were also able to produce *de novo* centrioles (Marshall et al., 2001).

In summary, these last studies claim the existence of two major centriole assembly modes, a template and a *de novo* pathway, proving that centrioles can be formed *de novo* in dividing cells that normally always contain template-formed centrioles and is not limited to specialized cases.

Nevertheless, despite of these pioneer studies on centriole assembly, the *de novo* pathway is less well understood and documented than is the template pathway, and some questions remain to be addressed - *"Is the de novo assembly pathway under the same cell cycle control as normal duplication?"*, *"How far these two modes of centriole assembly are fundamentally different or represent variations of a common pathway?"*. To answer these questions, two recent studies (Peel et al., 2007; Rodrigues-Martins et al., 2007b) started to unveil the molecular mechanism underlying *de novo* centriole assembly and its relationship with the template or canonical pathway. Peel et al. (2007) have demonstrated that overexpressing GFP-fusions of three centriole assembly specific proteins (PLK4,

DSas-6 and DSas-4) in *Drosophila* unfertilized eggs induced large numbers of *de novo* centriole-like structures that organized MTs and co-stained either with centriolar and centrosomal markers such as D-PLP, Cnn and γ-tubulin. Accordingly, this last study was complemented by another one conducted by Rodrigues-Martins et al. (2007b) in which overexpression of PLK4/SAK in *Drosophila* unfertilized eggs and embryos led to amplification of the pre-existing centrioles inherited from the sperm (also demonstrated by Peel et al., 2007) whereas it triggered *de novo* assembly of multiple centrioles throughout the cytoplasm in unfertilized eggs. Strikingly, in contrast with Peel and co-workers (2007), the *de novo* formed centrioles after PLK4 overexpression in unfertilized *Drosophila* oocytes were able to enter new rounds of centriole duplication through the canonical mode. These two reports revealed that proteins typically associated with templated centriole biogenesis also act to trigger *de novo* centriole assembly, suggesting a unique and common set of regulatory proteins between *de novo* and templated assembly modes.

As alternative sequences between three nucleotides of DNA can code for the same amino acid, the same protein module in a different context leads to the formation of centrioles. Therefore, centriole biogenesis appears as a potential redundant process in which there are two main "roads" based on the same molecular elements that culminate in one goal - centriole assembly. Nonetheless, it is also not possible to rule out the hypothesis of two common pathways that coexist but the template pathway normally suppresses the *de novo* pathway (Marshall et al., 2001; Nigg, 2007). Moreover, these last studies challenge the idea of the role played by the older centriole as a template on centriole biogenesis. Thus, *"If centrioles can be formed de novo, why do new centrioles form next to preexisting ones in most proliferating somatic cells?"*. This question will be discussed in the following section.

6.1. The Role of the Parental Centriole

Centriole duplication follows two simple rules: just one daughter centriole must arise per mother centriole once per cell cycle. Taking into consideration the previous reports, it is clear that *de novo* centriole assembly leads to a high and variable number of centrioles. Since supernumerary centrosomes are directly involved in multipolarity which in turn has been linked to genome instability, aneuploidy and ultimately cancer (Nigg and Raff, 2009), it is mandatory a strict control over centriole number.

Given that *de novo* centriole assembly seems not to occur when a resident centriole is present, even under overexpression of centriolar proteins such as SAS-6 and PLK4 in embryos and somatic cells (Peel et al., 2007; Rodrigues-Martins et al., 2007b), it might be possible that the centriole itself has the ability to suppress *de novo* centriole pathway and consequently, control the number of centrioles per cell cycle. Thus, the

mother centriole appears as an intermediate that contribute to the precise spatial, temporal and numerical control over centriole duplication during cell cycle. La Terra et al. (2005) proposed a model in which the mother centriole contains a "docking site" at the proximal end that stabilizes and accelerates procentriole formation. In this regard, it might be possible that during centriole duplication in each cell cycle, *de novo* centriole formation may occur, but only those procentrioles that associate with the mature/mother centriole are stabilized and prone to follow the next stages of centriole assembly. In opposition to the "docking site" theory, the mother centriole might also inhibit *de novo* centriole formation elsewhere in the cell through the integration of cellular signals. It may be possible that these two last mechanisms are not mutually exclusive and both can be used to control centriole assembly. Although their nature has not yet been truly understood, it might explain why *de novo* centriole assembly is blocked in centriole-containing cells.

Moreover, in all experimental systems previously described, either in *Chlamydomonas* mutant, unfertilized *Drosophila* oocytes or the laser ablation of centrosomes (Khodjakov et al., 2002; La Terra et al., 2005; Marshall et al., 2001; Rodrigues-Martins et al., 2007b), it was noticed that the kinetics of *de novo* centriole assembly was slower compared with the template pathway, which may imply the mother centriole as a platform that recruits and stabilizes centriole assembly promoting factors, hence offering a kinetic advantage to centriole formation by providing a surface or a "docking site" that favours the assembly reaction (Cunha-Ferreira et al., 2009; Nigg and Raff, 2009).

Another alternative lies on the interface between PCM and centrioles. Since within PCM resides a variety of regulatory factors important for centriole assembly (Dammermann et al., 2004), it is possible to speculate that the well-defined cloud of PCM surrounding each centriole may enhance procentriole assembly by ensuring a local concentration of essential duplication factors, where protein-protein interactions and activation are promoted (Loncarek et al., 2008; Loncarek and Khodjakov, 2009; Nigg and Raff, 2009). This last approach depends largely on the symbiotic relationship between mature centrioles and their own PCM, whereby the absence of a resident centriole would promote the self-assembly of PCM clouds throughout the cytoplasm to eventually give rise to *de novo* centriole formation foci. In agreement with this point of view, La Terra et al. (2005) showed that *de novo* centriole formation in acentrosomal HeLa cells was preceded by the accumulation of small centrin-GFP aggregates in the cytoplasm, some of them disappeared within a few hours after their formation. Thus, it is possible that lack of centrosome or a resident centriole prevents the efficient transport of these small PCM pieces to the centrosome and, therefore prevents the spatial restriction of centrosome

assembly to the vicinity of the mother centriole. However, future work is needed to better understand these issues.

In summary, the last studies showed that centriole assembly can be a template-free process, regardless of the cell type.

In spite of the last and diverse speculations about the origins and control of *de novo* centriole assembly, it should be kept in mind that these two pathways of centriole assembly seem to display common molecular requirements, thus they probably are not so different as we may think. Loncarek and Khodjakov in *"Ab ovo or de novo? Mechanisms of centriole duplication"* (2009) raised pertinent questions such as: *"Is canonical centriole duplication a spatially-restricted and more stringently controlled version of centriole de novo assembly?*; *"If the template centriole duplication provides far more precise control over centriole numbers, then why do so many organisms employ centriole de novo formation, in such critical events as early embryogenesis ?"*. These questions of centriole biogenesis are begging for more answers and its comprehension might help to understand the dichotomy between extra centrosomes and cancer.

OBJECTIVE

Aim of this Project

Acentriolar systems represent a powerful source to study the modalities and the machinery involved in the acentrosomal microtubule assembly pathways as well as to address fundamental questions concerning centriole functions and biogenesis.

Previous works using unfertilized *Drosophila* oocytes and mammalian somatic cells as experimental systems have shown that *de novo* centriole biogenesis can be induced in the absence of the resident centriole, even in cells that would rely on a template or canonical mode for centriole formation. However, this fundamental question about centriole biogenesis was still not addressed in somatic cells born without centrioles.

Taking advantage of a newly *Drosophila melanogaster* acentriolar cellular line (*DSas-4 -/-* # 131), this project ‘s major goal was to investigate the occurrence of *de novo* centriole biogenesis in somatic *Drosophila melanogaster* acentriolar *DSas-4 -/-* cells through the reintroduction of the *Drosophila* SAS-4 protein. Therefore, this work is divided in three parts:

1. Characterization of the acentriolar *DSas-4 -/-* cells regarding the expression/localization of centriolar and PCM proteins and mitotic spindle morphology;

2. Recombinant DSas-4 protein expression optimization;

3. Analysis and characterization of the *DSas-4 -/-* cells phenotype concerning the formation of *de novo* centrioles after DSas-4 reintroduction:

3.1. Localization and presence of centriolar proteins;
3.2. Number of *de novo* formed centrioles;
3.3. Protein expression analysis of centriolar and PCM components;
3.4. Microtubule nucleation ability through the presence of astral microtubules in mitosis;
3.5. Mitotic spindle morphology;
3.6. Electron microscopy analysis.

2. MATERIAL & METHODS

Cell Culture

Drosophila Jupiter and *DSas-4 -/-* cells were maintained at 25 ºC. Both cell lines were grown in M3 Shields and Sang medium (Sigma-Aldrich) supplemented with 10 % of heat inactivated fetal calf serum (FBS). The same growth conditions were used for the transfected Jupiter and *DSas-4 -/-* cells.

Population Doubling Time

Cells were plated in 6 well plates at final concentration of one million cells, and counted every 48h during one week. Population doubling time (PD) was determined using the following formula, $PD = T * \left(\frac{ln2}{\ln\left(\frac{xf}{xi}\right)}\right)$, in which T = timepoint (h), x_f = final concentration, x_i = initial concentration ($1x10^6$ cells/mL). The average of three replicates *per* timepoint was used as indicator of doubling time.

Constructs and BLAST cloning

Vectors under the control of a metallothionein promoter, pMT-DSas-4-mRFP construct was generated with the Gateway® system (Invitrogen) and pMT-DSas-4-GFP construct was kindly provided by Gohta Goshima (Nagoya University, Japan). Blasticidin coding gene was introduced into the previous constructs using the restriction enzyme SalI.

Transfections and generation of Stable cell lines

Transfection of Jupiter and *DSas-4 -/-* cells with GFP and mRFP tagged Sas-4 protein was performed using Effectene transfection reagent (Quiagen) according to the manufacturer instructions. Cells were first plated in 12 wells plates the day before transfection. Stable integrates were selected with 25 µg/ml blasticidin S (Invitrogen) during approximately 3-4 weeks. Stable cell lines were confirmed by western blot using an anti-GFP primary antibody.

Antibodies

The primary antibodies used in immunofluorescence were: mouse anti-γ-Tubulin 23C (1:200) (Sigma-Aldrich), rat anti-α-Tubulin MCA 77G (1:100) (AbD Serotec), mouse anti-α-Tubulin B512 (1:2000) (Sigma-Aldrich), rabbit anti-Asterless (1:500), guinea pig

anti-Ana1 (1:500), rabbit anti-D-PLP (1:500) (all gift from Jordan Raff, University of Oxford, UK), rabbit anti-centrosomin (Cnn) (1:2000), rabbit anti-Sas-4 (1:500) (all gift from Renata Basto, Institute Curie, France), chicken anti-Sas-6 (1:200) (gift from Mónica Bettencourt-Dias, Gulbenkian Institute, Portugal), rabbit anti-mRFP (Invitrogen) and rabbit anti-GFP. The primary antibodies were detected with the respective secondary antibodies conjugated with Alexa-488, 568 or 647 (1:1000) (Invitrogen, Molecular Probes).

Immunofluorescence analysis

Cells were plated overnight on Concanavalin-A (Calbiochem) coated glass coverslips in M3 medium. For the transfected DSas-4-mRFP cell lines, induction periods of 24-96 hours at 0.5 and 1mM $CuSO_4$ were performed. Expression of DSas-4-GFP was induced over periods of 24-72 hours using the former copper concentrations. Cells were fixed in cold methanol for 5 minutes at -20 ºC followed by a permeabilization step with 0.3 % Triton X-100 in cytoskeletal buffer for 3 minutes and rinsed 3x5 minutes in PBS + 0.05 % TWEEN® 20 (Sigma-Aldrich). Exceptionally for DSas-4 staining, DSas-4-mRFP transfected cell lines and in figure 10, it was required a fixation of 10 minutes with 4 % paraformaldehyde in cytoskeletal buffer at room temperature followed by a cell permeabilization of 10 minutes in 0.3 % Triton X-100 and rinsed 3x5 minutes in PBS. Cells were blocked between 1-2 hours in PBS + 0.05 % TWEEN® 20 containing 10 % FBS. After blocking, cells were then incubated overnight with primary antibodies diluted in the blocking solution and washed 3x5 minutes with PBS + 0.05 % TWEEN® 20. DNA was counterstained with DAPI (1:100 000) and the preparations mounted in mounting medium. Immunofluorescence images were collected using an AxioImager Z1 microscope with an AxiocamMRm CCD. Images were collected using a 100X, 1.4 numerical aperture (NA) oil immersion objective with 0.25 µm z-sections and subsequently deconcolved in AutoQuant X (MediaCybernetics). All images were prepared with Adobe Photoshop and ImageJ.

Quantification of protein fluorescence intensity

To determine protein fluorescence intensity, sum slices projections of z-stacks covering the entire mitotic spindle poles were generated. Protein fluorescence intensity was performed by selecting the centrosome or protein aggregates at pole regions using a define ROI of ImageJ (National Institutes of Health) and measuring the total fluorescence intensity in the entire z-stacks. The resulting value was normalized to the corresponding area.

Western Blotting and Inducible Expression of DSas-4-GFP

For the induction of DSas-4-GFP expression in the stable cell lines, cells were incubated in M3 medium containing 1mM $CuSO_4$ and collected after 24-72 hours of induction. For the parental Jupiter and *DSas-4 -/-* cells the same procedure was used. Cells were harvested by centrifugation and washed once with PBS. Protein extracts were obtained by resuspending the cell pellet in lysis buffer NP-40 containing 2mM DTT, 1mM PMSF and 1x Proteases Inhibitor at 4ºC for 30 min. Samples were centrifuged at 4ºC and the supernatant was collected for subsequent protein quantification with Bradford assay. Standard procedures were used for Western blotting. Gels differing in polyacrylamide concentration were used to resolve different proteins (DSas-4-GFP [7.5%], Asterless, Cnn, DSas-4 [8%] and DSas-6 [12%]). Primary antibodies were incubated overnight at 4ºC and secondary antibodies during 1-1.30 hours at room temperature in 5-10% milk. The primary antibodies used in western blotting were: rabbit anti-Asterless (1:1000), mouse anti-DSas-4 (1:1000) (all gift from Jordan Raff, University of Oxford, UK), rabbit anti-Cnn (1:2000) (gift from Alain Debec, Institute Jacques Monod, France), chicken anti-DSas-6 (1:500) (gift from Mónica Bettencourt-Dias, Gulbenkian Institute, Portugal), rabbit anti-GFP (1:100), mouse anti-α-Tubulin B512 (1:5000) (Sigma-Aldrich), goat anti-actin sc-1616 (1:2000) (Santa Cruz Biotechnology). Appropriate secondary antibodies were used for detection using ECL (Pierce).

Live cell imaging

Drosophila DSas-4 -/- rescued cells were induced for 48h at 1mM CuSO4 and plated the day before live imaging in Ibidi glass bottom dishes (Ibidi) pre-coated with 0.25 mg/ml concanavalin A.

Live-cell imaging was performed in a temperature-controlled Nikon TE2000 microscope equipped at the camera port with a modified Yokogawa CSU-X1 spinning-disc head (Solamere Technology), an ASI FW-1000 filter wheel followed by the Andor iXon+ DU-897E EM-CCD. The excitation optics is composed of two Coherent Sapphire lasers at 488nm and shuttering is performed by a Gooche&Housego R64040-150 acousto-optic tunable filter. Sample position is controlled via a SCAN-IM Marzhauser stage and a Physik Instrumente 541.ZSL piezo. A 100x Plan-Apo DIC CFI Nikon objective were used in all experiments, with a 2min time-lapse and 1 μm step image stacks.

Electron Microscopy

EM was performed according to Lecland et al. (2013).

3. RESULTS

PART 1

PART 1

1. Characterization of the *Drosophila* Acentriolar *DSas-4 -/-* cell line # 131

1.1. Acentriolar Mitotic Spindle Assembly

The acentriolar nature of *DSas-4 -/-* cells comes from the insertion of a P-element that truncates the DSas-4 protein, therefore impairing centriole biogenesis (Basto et al., 2006). This cell line was derived from dissociated DSas-4^{S2214} embryos (aged from 3 to 14 hours), also bearing the *Jupiter-GFP* protein in the mutant allele (Lecland et al., 2013).

In order to study the overall spindle morphology of *DSas-4 -/-* acentriolar mitotic cells, an immunofluorescence protocol was used and cells were stained with α-tubulin and DAPI, for visualization of the mitotic spindle and chromosomes, respectively. Although, the *DSas-4 -/-* mutant cell line constitutively expresses the fusion protein Jupiter-GFP that marks the MT array of the mitotic spindle, the α-tubulin staining was required to improve the visualization of the microtubules. Importantly, for all the experiments the *Drosophila* Jupiter cell line containing centrosomes was used as control (Karpova et al., 2006). During mitosis, five types of acentriolar mitotic spindles were reported in a previous work characterizing this acentriolar cell line (Lecland et al., 2013) (Fig. 5, H). Accordingly, the acentriolar nature of the *DSas-4 -/-* cells can lead to wild-type like, barrel, dual, hyperpolarized or non-polarized mitotic spindle morphological types (Fig. 5, A-F). Specifically in this study, one more anastral spindle category (Undetermined) was included representing the cells that did not fit in any of the other categories.

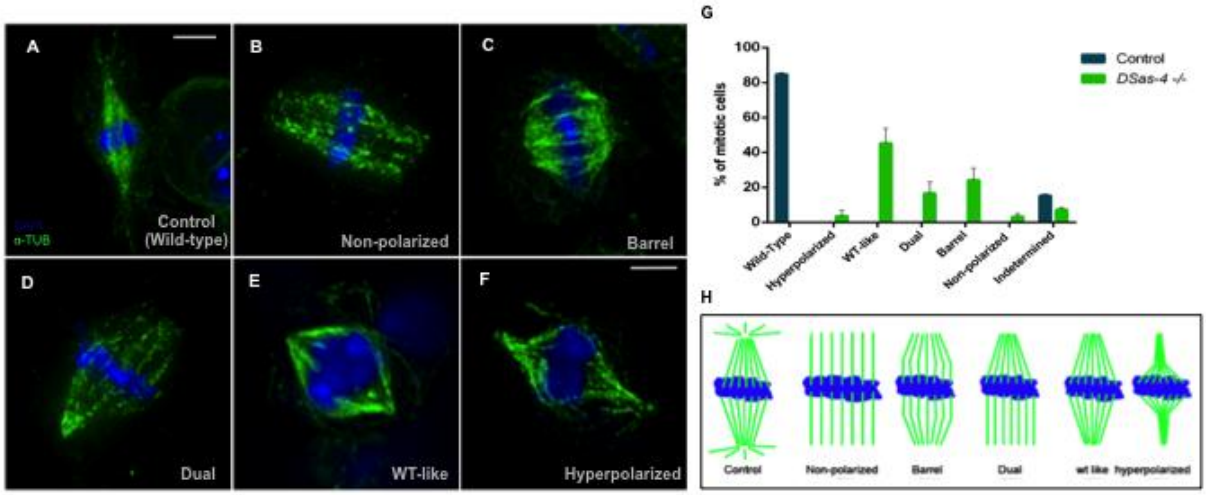


Figure 5. Characterization of spindle morphology in control and *DSas-4-/-* mutant cells. (A-F) Immunofluorescence on fixed cells. (A) Control cell exhibiting a polarized biconical mitotic spindle with astral MTs around the centrosome in both poles. (B-F) Representative acentriolar spindle

shapes identified in *DSas4 -/-* mutant cells. These ranged from more focused to non-polarized spindles. Scale bar, 5 µm. **(G)** Percentage of mitotic cells *per* category of spindle morphology in control cells: WT (85%) and Undetermined (15%); and in the acentriolar cell line: Barrel (24%); Dual (16.6%); Hyperpolarized (3.6%); WT-like (45%); Non-polarized (3.2%) and Undetermined (7.3%). A total number of control cells (n=85) from two independent experiments and *DSas-4 -/-* cells (n=438) from three independent experiments were analyzed. Error bars represent SD. **(H)** Categories of spindle morphology in control and acentriolar mitotic cells according Lecland et al., 2013.

In the control cell line approximately 85% of cells exhibited the usual bipolar configuration with astral MTs surrounding each centrosome characteristic of a typical centrosomal mitotic spindle, compared with just 15% of cells that had an undetermined spindle shape, probably reflecting incomplete spindle assembly (Fig. 1, G). On the contrary and as mentioned before, the acentriolar *DSas-4 -/-* cells showed a variety of non-centrosomal spindle shapes. The barrel-shaped spindle is usually poorly polarized, with broad poles whereas the WT-like spindle exhibits a normal polarization. The dual spindle shows an asymmetry between poles, in which only one of the poles is focused. The hyperpolarized spindle shows an extreme polarization and the non-polarized spindle exhibits no focalization of the spindle extremities (Fig. 5, B-F). In this study, the predominant spindle morphology was the WT-like, which was present in approximately 50% of the mitotic cells, followed by the barrel (24%) and the dual (16%) shapes. The less frequent were the hyperpolarized and the non-polarized with 4% and 3% of cells respectively, and just 7% of cells had an undetermined spindle shape (Fig. 1, G). In all morphological spindle types no centrosome-like structures or astral MTs were detected at the poles. Despite undetectable astral MTs, these cells showed robust K fibers and interpolar MTs (Fig. 5, B-F).

1.2. Recruitment of PCM and Centriolar Proteins in *DSas-4 -/-* cells

In order to analyze the localization of PCM and centriolar proteins in control and *DSas4 -/-* mutant cells, centrosomin and D-PLP antibodies were used. In control cells, D-PLP is present at the center of the centrosome and Cnn localizes to the centrosomes of metaphase cells (Fig. 6, upper panel). In *DSas-4 -/-* cells both proteins can be detected as disperse spots of variable size at the broad poles of the acentriolar mitotic spindles (Fig. 6, lower panel). In order to analyze the quantity of Cnn and D-PLP pool recruited to the acentriolar poles of *DSas-4 -/-* cells in comparison to control cells, fluorescence intensity

was measured for each protein, and co-localizations between both proteins was taken into account to avoid the measurement of unspecific dots. The mean fluorescence pixel intensity of Cnn and D-PLP at the centrosomes of control cells was 2.39 ± 1.21 and 1.47 ± 1.07 respectively, whereas *DSas-4 -/-* cells revealed a reduction in the fluorescence intensity of approximately 90 % for D-PLP and 70 % for Cnn (Fig. 6, B and C).

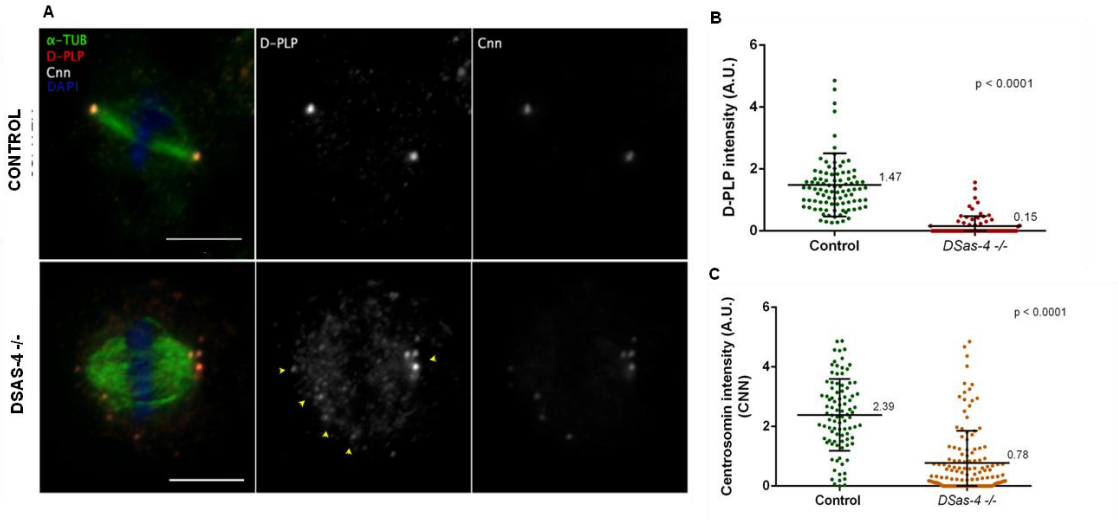


Figure 6. Cnn and *Drosophila* Pericentrin-like (D-PLP) accumulate at mitotic spindle poles of acentriolar *DSas-4 -/-* cells. (A) Immunofluorescence on fixed cells. Control (upper panel) and *DSas-4 -/-* cells (lower panel) were stained for the centriolar and PCM markers D-PLP (red), Cnn (white), DNA (blue) and α-tubulin (green). The yellow arrowheads in the lower panel highlight D-PLP and Cnn co-localizations in *DSas-4 -/-* line. Scale bar, 10 μm. **(B)** D-PLP absolute fluorescence intensity levels with an average of 1.47 in control cells and 0.15 in *DSas-4 -/-* cells. **(C)** Cnn absolute fluorescence intensity levels with an average of 2.39 in control cells and 0.78 in *DSas-4 -/-* cells. Note that only the fluorescence intensity of Cnn and D-PLP co-localizations were measured and counted. Each dot in control cells represents one centrosome and in *DSas-4 -/-* cells represents foci of CNN and D-PLP accumulations. Cells with extra centrosomes in control line were not analyzed. Error bars represent SD and mean. Statistically significant differences (Student's t-test) from the respective controls are shown ($p<0.0001$); a total number of control (n=50) and *DSas-4 -/-* (n=66) mitotic cells were analyzed from one experiment.

Since it was verified the presence of Cnn and D-PLP proteins at the poles of acentriolar mitotic spindles, we then investigated the localization of other centriolar components. The first analysis was performed with Asterless (Asl), an essential protein for centriole formation in *Drosophila* and also required for recruitment of PCM (Dzhindzhev et

al., 2010; Varmark et al., 2007). In wild-type cells, Asl is present as one define dot at each side of the metaphase plate localizing to the centrioles (Fig. 7, upper panel). In the acentriolar cell line, similar to the recruitment of Cnn and D-PLP, Asl can be detected as small dots of variable size at the acentriolar poles of *DSas-4 -/-* cells during mitosis (Fig. 7, lower panel). Also, by measuring the fluorescence intensity levels of Asl accumulation, a reduction of approximately 70 % (Fig. 7, B) in its polar recruitment, compared with control cells (mean= 1.52 ± 0.632), was verified in acentriolar spindles.

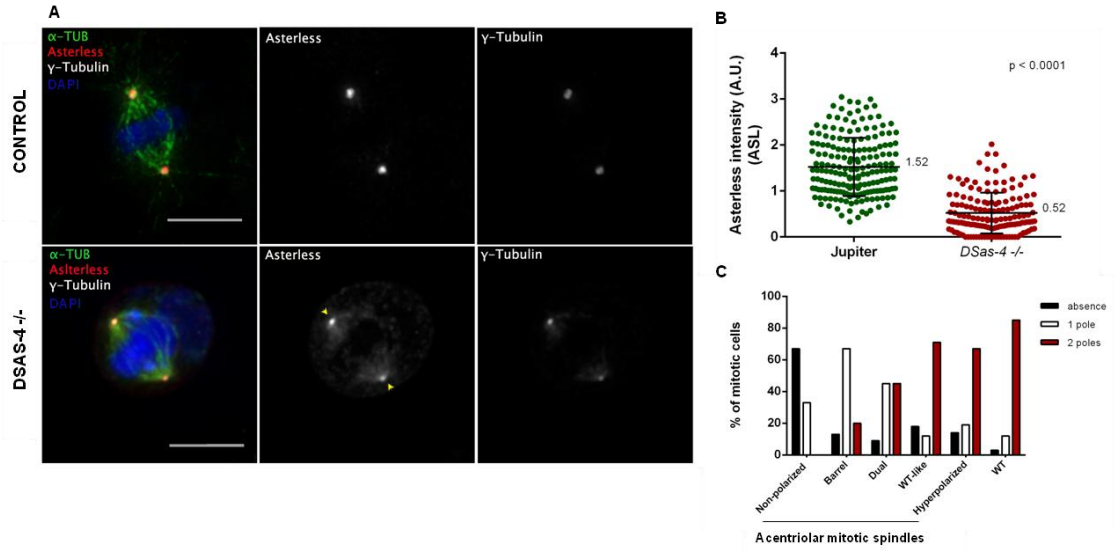


Figure 7. Asterless (Asl) accumulate at mitotic spindle poles of acentriolar *DSas-4 -/-* cells. (A) Immunofluorescence on fixed cells. Control (upper panel) and *DSas-4 -/-* cells (lower panel) were stained for Asl (red), for the centrosomal marker γ-tubulin (white), DNA (blue) and α-tubulin (green). The yellow arrowheads in the lower panel highlight Asl accumulations and respectively co-localization with γ -tubulin in *DSas-4 -/-* line. Scale bars, 10 μm. **(B)** Asl absolute fluorescence intensity levels with an average of 1.52 in Jupiter control cells and 0.52 in *DSas-4 -/-* cells**.** Note that only the fluorescence intensity of Asl accumulations in co-localization with γ-tubulin was measured and quantified. Each dot in control cells represents one centrosome and in *DSas-4 -/-* cells represents foci of Asl accumulations. Cells with extra centrosomes in the control cell line were not analyzed. Error bars represent SD and mean. Statistically significant differences (Student's t-test) from the respective controls are shown ($p<0.0001$); a total number of control (n=90) and *DSas-4 -/-* (n=90) mitotic cells were analyzed from one experiment **(C)** Relationship between Asl accumulations at the spindle poles of *DSas-4 -/-* cells with the type of acentriolar mitotic spindle. Black bars represent absence of Asl foci in both poles, white bars represent the presence of Asl foci just in one spindle pole and red bars represent the presence of Asl foci in the two spindle poles. Absence: Control (3%); non-polarized (67%); Barrel-shaped (13%); Dual (9%); WT-like (18%); hyperpolarized (14%). 1 pole: Control (12%); non-polarized (33%); Barrel-shaped (67%); Dual (45%); WT-like (12%); hyperpolarized (19%). 2 poles: Control (85%); non-polarized (0%);

Barrel-shaped (20%); Dual (45%); WT-like (71%); hyperpolarized (67%). A total number of control (n=93) and *DSas-4 -/-* (n=90) mitotic cells were analyzed from one experiment.

Additionally, it was addressed the relationship between Asl polar accumulations in the acentriolar cell line with the different morphologies of the acentriolar mitotic spindle. Thus, for each spindle morphological category, cells were classified accordingly with the presence (in one pole or at both poles) or absence of Asl. As expected, in 90 % of bipolar mitotic spindle in control cells, Asl was present at both spindle poles (Fig. 7,C). In *DSas-4 -/-* mutant cells, it was verified that approximately 70 % of mitotic cells with WT-like and hyperpolarized acentriolar spindles showed Asl foci at both poles. The mitotic spindles with dual morphology showed a divided phenotype where in 45 % of the cases Asl foci were present in both poles, and in the other 45% Asl localization was confined to only one of the poles. On the other side, in 67 % of mitotic barrel-shaped cells, Asl spots were present in just one of the two spindle poles. The most striking phenotype was observed in cells exhibiting a non-polarized spindle where 67 % of cases showed no Asl accumulations at the spindle poles (Fig. 7, C). It was possible to note that the PCM protein *Drosophila* γ-tubulin 23C also localized in the acentriolar poles of *DSas-4 -/-* cells and stained to the mitotic spindle (Fig. 7, A, lower panel). Similarly, both Cnn, D-PLP and Asl also localized over the mitotic spindle in *DSas-4 -/-* cells (Fig. 6 and fig. 7, A, lower panel).

To specifically confirm whether the acentriolar spindles in the *DSas4 -/-* cell line were devoid of centrioles, the localization of specific centriolar proteins, such as Ana1 and DSas-6 was also investigated. *Drosophila* Sas-6 is permanently associated with the proximal end of centrioles localizing to the cartwheel (Mennella et al. 2012; Rodrigues-Martins et al. 2007; Van Breugel et al. 2011) and Ana1 has been reported to be associated with centrioles in S2 cells and *Drosophil*a embryos as well as with the basal body in *Drosophila* spermatids and sensory neurons (Blachon et al. 2008; Blachon et al. 2009; Dobbelaere et al. 2008; Gopalakrishnan et al. 2011; Goshima et al. 2007). In contrast with Cnn, D-PLP, Asl and γ-tubulin stainings, there was no polar accumulation of Ana1 (Fig. 8, A, lower panel) or DSas-6 (Fig. 8, C, lower panel) in the anastral mitotic spindles of mutant cells and consequently these proteins showed a dispersed localization in the cytoplasm. To further confirm the data from fixed cell analysis, the number of Ana1 and DSas-6 polar foci were quantified in control and in DSas4 mutant cells (Fig. 8, B and D). In 100 % of the mutant cells, Ana1 and DSas-6 were absent from the poles, compared with approximately 90 % of control cells in which these proteins were recruited to the centrosomes.

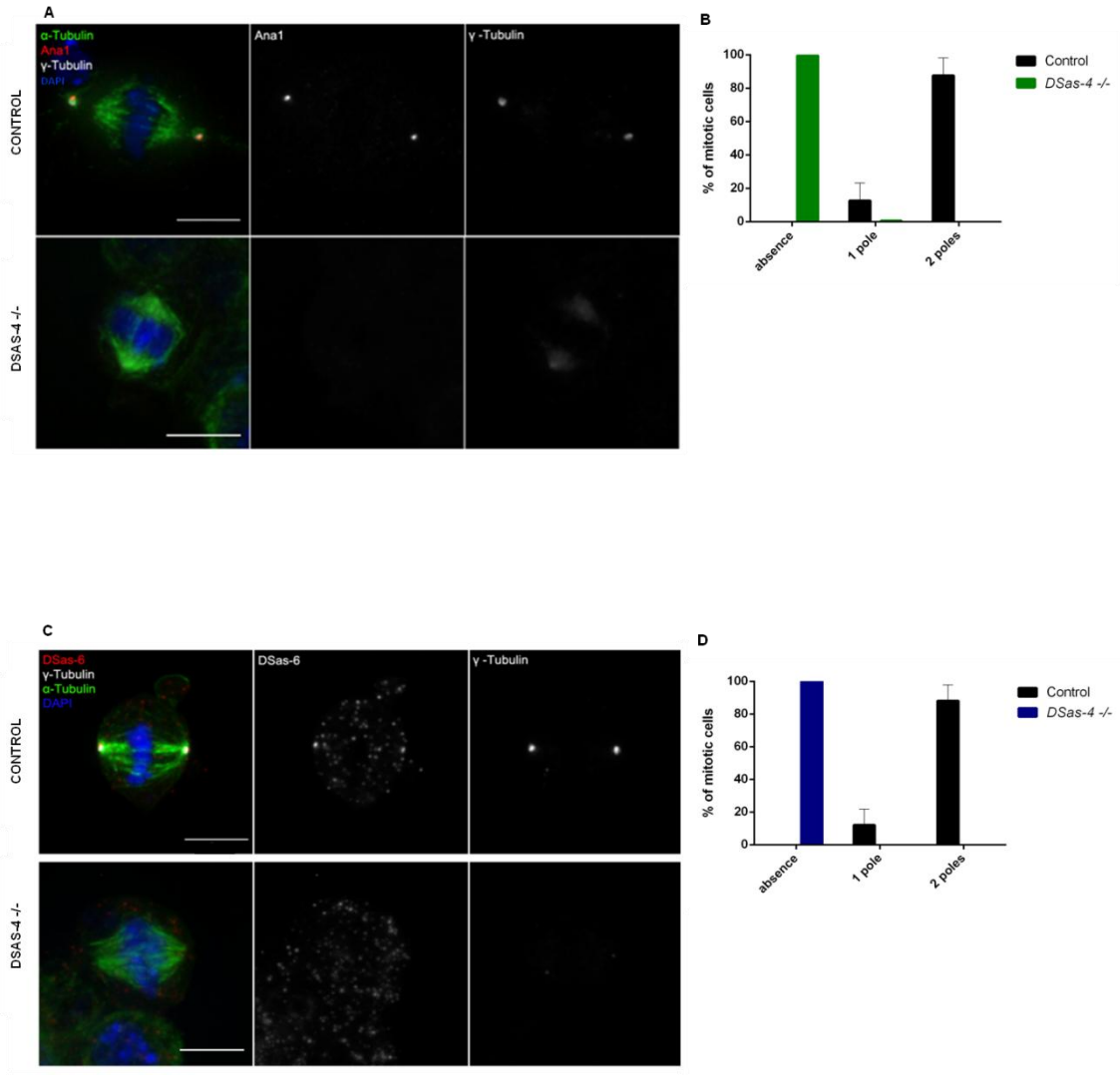


Figure 8. Ana1 and DSas-6 are not recruited to the acentriolar mitotic spindle poles of *DSas-4 -/-* cells (A) Immunofluorescence on fixed cells. Control (upper panel) and *DSas-4 -/-* cells (lower panel) were stained for the centriolar marker Ana1 (red), centrosomal marker γ-tubulin (white), DNA (blue) and α-tubulin (green). **(B)** Percentage of mitotic cells with Ana1 presence (at one pole or both poles) or absence in control and *DSas-4 -/-* cells. Absence: control (0%); *DSas-4 -/-* (99%). 1 pole: control (12.5%); *DSas-4 -/-* (1%). 2 poles: control (87.5%); *DSas-4 -/-* (0%). A total number of control (n=61) and *DSas-4 -/-* (n=141) mitotic cells were counted in two independent experiments. **(C)** Immunofluorescence on fixed cells. Control (upper panel) and *DSas-4 -/-* cells (lower panel) were stained for the centriolar marker DSas-6 (red), centrosomal marker γ-tubulin (white), DNA (blue) and α-tubulin (green). Scale bars in (A) and (C), 10 μm. **(D)** Percentage of mitotic cells with DSas-6 presence (at one pole or both poles) or absence in control and *DSas-4 -/-* cells. Absence: control (0%); *DSas-4 -/-* (100%). 1 pole: control (12%); *DSas-4 -/-* (0%). 2 poles: control (88%); *DSas-4 -/-* (0%). A total number of control (n=46) and *DSas-4 -/-* (n=37) mitotic cells were counted in two independent experiments. Error bars from each graph represent the SD.

From these experiments, we conclude that *Drosophila* SAS-4 mutant cells lack functional mitotic centrosomes due the absence of centrioles. The recruitment of centriole-specific proteins to the mitotic poles is affected, and there is variation on the morphology of the mitotic spindle due to the lack of a centrosome acting as MTOC.

PART 2

Part 2

2. Transfection of DSas-4 protein in the *DSas-4 -/-* cells

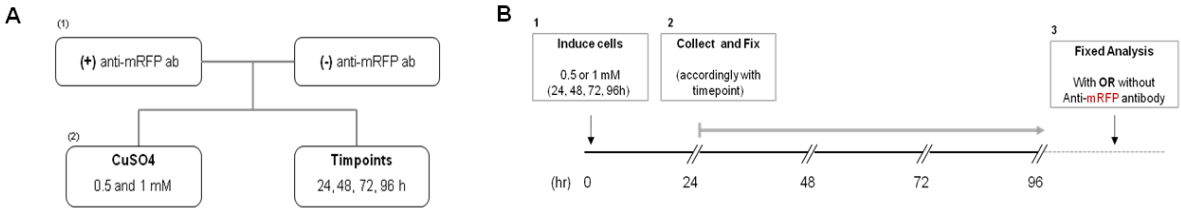
2.1. Optimization of DSas-4 protein expression

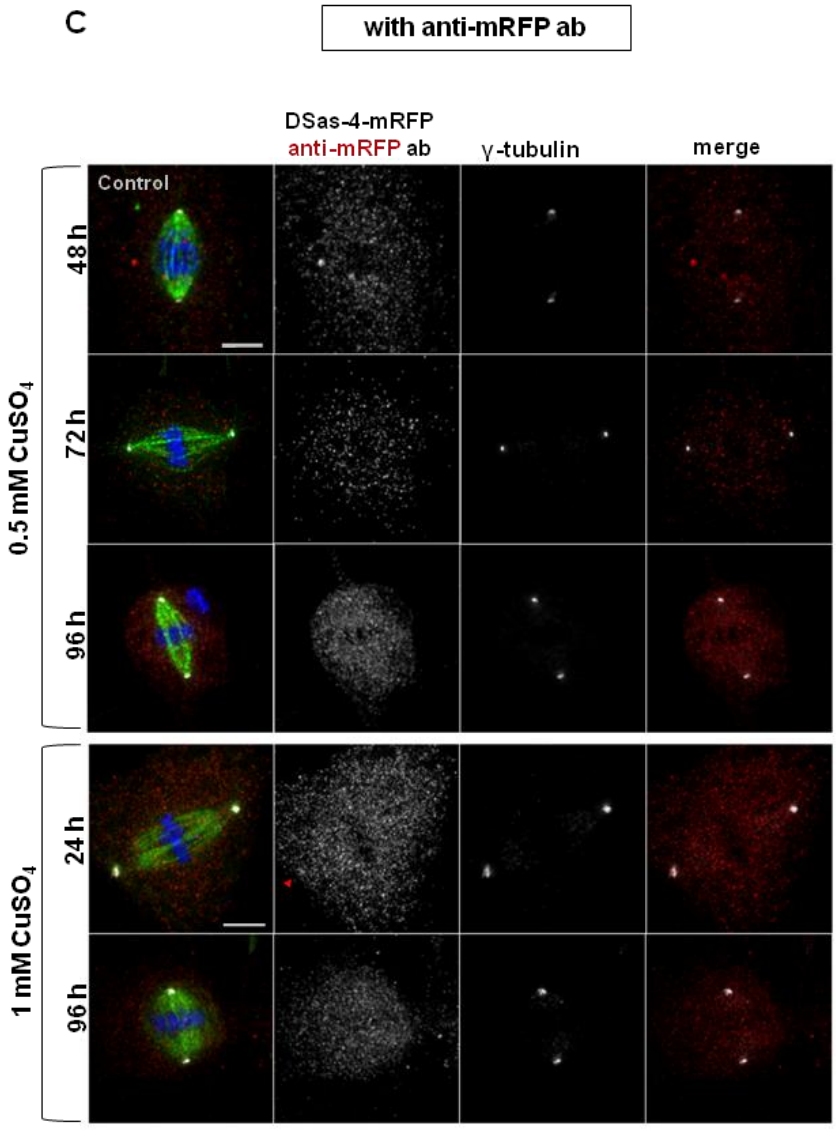
2.1.1. DSas-4-mRFP construct

The main goal of this study was to assess *de novo* centriole formation in somatic acentriolar *DSas-4 -/-* cells by SAS-4 protein reintroduction in an attempt to better understand the dichotomy between canonical/template and *de novo* centriole formation in dividing cells. In order to rescue the acentriolar phenotype of the mutant cell line, the first goal of this study was to successfully transfect the *Drosophila* SAS-4 protein in *DSas-4 -/-* cells. With this goal in mind, stable cell lines were generated carrying a full-length DSas-4-mRFP fusion protein under the control of the metallothionein promoter. The expression of DSas-4-mRFP protein was first examined and optimized with two approaches: 1) by transiently transfecting the DSas4-mRFP construct in *Drosophila* S2-U cells and 2) by stably transfecting the DSas4-mRFP construct in the control cell line previously used for comparison with the DSas4 mutant cell line. The analysis of the positively transfected cells was done by immunofluorescence on fixed cells. Since the *Drosophila* S2-U cells are easily transfected, they were firstly used in order to verify the functionality of the plasmid. After the transient transfection and induction of the DSas4-mRFP construct during 24h and 48h with the standard copper concentration used for *Drosophila* S2-U cells (0.5 mM), it was possible to detect the fusion DSas-4-mRFP signal at centrioles as two bright dots in a high proportion of S2-U cells (Fig. 9, E). Then, the conditions used for *Drosophila* S2-U cells were also applied to the control cell line used in this study. However the direct detection of DSas-4-mRFP signal at centrioles in the transfected control cell line was not successful (data not shown).

Therefore, a protein expression optimization protocol was elaborated, and several conditions taking into account the induction time and copper concentration were set out (Fig. 9, A and B). A polyclonal antibody raised against mRFP (monomeric red fluorescent protein) was used in order to improve the detection of the exogenous DSas-4-mRFP fusion protein. The experimental scheme used for DSas-4-mRFP expression optimization is shown below (Fig. 9, B). As demonstrated in figure 9C and 9D, independently of the copper concentration (0.5 or 1 mM), the induction time (48, 72, 96 h) or the presence or not of the anti-mRFP antibody, the DSas-4-mRFP signal was not detected at centrioles in the majority of the control cells in the stable cell line through all stages of the cell cycle. Yet, in a small proportion of cells a faint DSas-4-mRFP signal was present at centrioles, as confirmed by co-localization with γ-tubulin 23C (Fig. 9, C and D, red arrowheads).

Nevertheless, either due to the unsuccessful detection of DSas-4-mRFP protein in majority of stable transfected control Jupiter cells or due to its faint presence, the created stable cell lines expressing DSas4-mRFP were not used for the purpose of this study. Interestingly, the expression of a full-length recombinant DSas-4-mCherry protein in control cells (Jupiter) also resulted in a failure to detect DSas-4 at centrosomes through fixed cell analysis similar to the DSas-4-mRFP construct (data not shown).





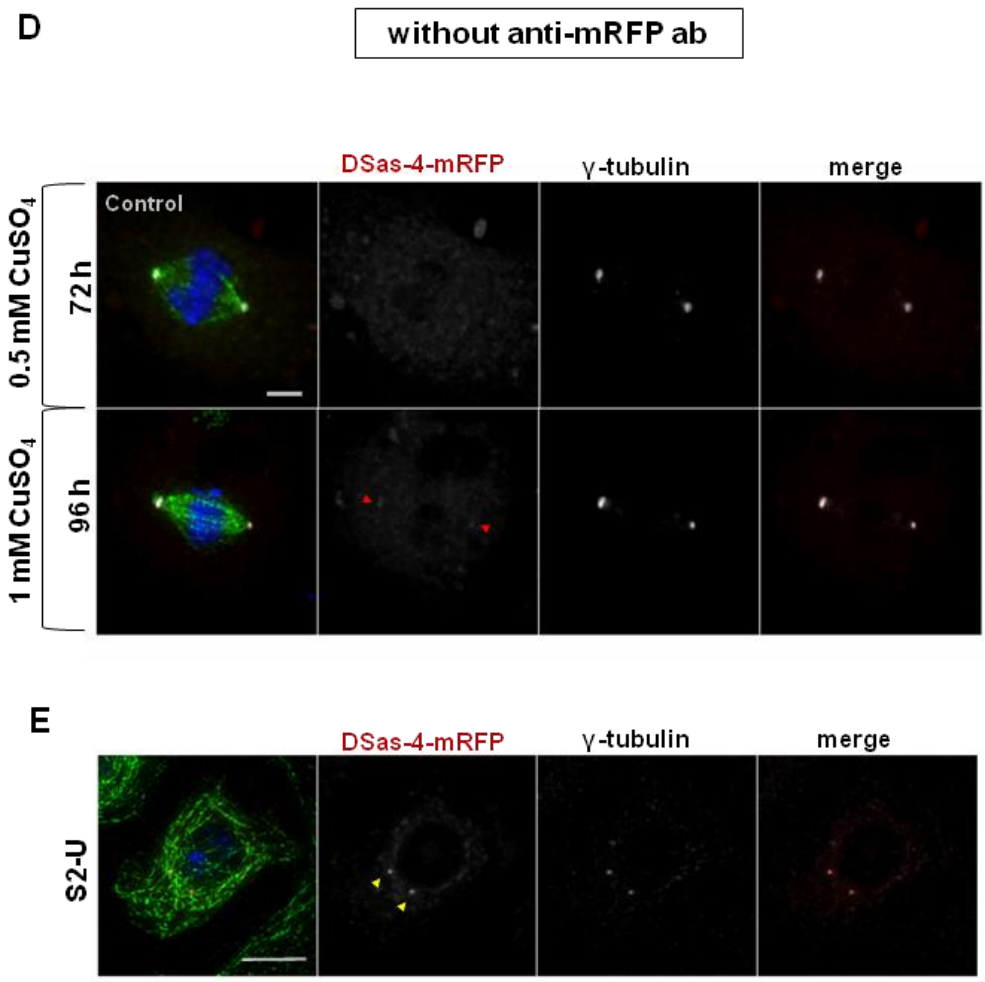


Figure 9. mRFP fusion of DSas-4 protein was not detected at centrioles in Jupiter control stable cell line. (A) and **(B)** Schematic outlines of the experimental strategy used for DSas-4-mRFP optimization. (A) Optimization conditions. (1) (+) and (-): with or without anti-mRFP antibody. (2) Copper sulfate ($CuSO_4$) concentrations and timepoints. Note that each cooper concentration was tested individually in all timepoints. (B) Experimental scheme representing the four experimental stages executed for the analysis of the DSas-4-mRFP expression by immunofluorescence. 1. Cells were plated and induced with 0.5 or 1 mM of $CuSO_4$ to allow the expression of the transfected version of DSas-4 protein; 2. At each time point cells were collected and fixed; 3. Cells were processed for immunofluorescence analysis and the detection of exogenous DSas-4 protein was examined, using or not the anti-mRFP antibody, co-stained with γ-tubulin 23C. **(C)** Immunofluorescence analysis of Jupiter control cells with anti-mRFP antibody to detect DSas-4-mRFP fusion. Not all the conditions (according with the optimization plan) are shown **(D)** Immunofluorescence analysis of Jupiter control cells with direct detection of DSas-4-mRFP fusion protein. In (C) and (D) the red arrowheads point to a potential DSas-4-mRFP signal at centrioles **(E)** Immunofluorescence analysis of *Drosophila* S2-U cells transiently expressing the fusion protein DSas-4-mRFP (24h, 0.5 mM). Note that in this specific case the transfection signal

was detected without an anti-mRFP antibody. The yellow arrowheads highlight the DSas-4-mRFP signal at the centrosomes of an interphasic cell co-localizing with the centrosomal marker γ-tubulin 23C. In all experiments cells were stained for α-tubulin (green), DNA (blue), DSas-4-mRFP or anti-mRFP ab (red) and γ-tubulin (white). Scale bars, 5 μm

2.1.2. DSas-4-GFP construct

Given that it was not possible to detect the fusion *Drosophila* Sas-4 protein signal in the control transfected stable cell line using the previous plasmid, a GFP-tagged protein version was examined. Therefore, a control Jupiter stable line carrying full-length DSas-4-GFP fusion protein under the control of the metallothionein promoter was generated. In order to optimize the expression conditions of the exogenous DSas-4-GFP protein, similar optimization parameters used for the detection of mRFP-tagged DSas-4 protein were tested. In this specific case, only three timepoints (24, 48 and 72h) for each copper concentration (0.5 and 1 mM) were examined. In order to evaluate the expression levels of DSas-4-GFP for each copper concentration and induction time, a comparative protein expression analysis by western blotting using an antibody raised against GFP (green fluorescent protein) was performed. It should be noted that, in spite of the presence of the endogenous DSas-4 protein in control cells, the anti-GFP antibody only detects the exogenous DSas-4 protein. As shown in figure 10A (right column), there was no presence of DSas-4-GFP protein expression in control and *DSas-4 -/-* parental lines. The absence of the GFP-tagged version of DSas-4 protein in the previous cells was completely expected since both cell lines were not transfected. In contrast, addition of 0.5 or 1 mM of $CuSO_4$ to the medium, induced the expression of DSas-4-GFP fusion protein in the control cells, thereby confirming the successful establishment of a transfected control cell line stably expressing the exogenous DSas-4-GFP protein. A qualitative analysis comparing DSas-4-GFP expression levels between each condition revealed that DSas-4-GFP expression increased from 0.5 mM to 1 mM of $CuSO_4$, suggesting that increased copper concentration influences the quantity of exogenous DSas-4-GFP protein being expressed. On the other side, the addition of 0.5 mM of copper did not alter significantly the DSas-4-GFP expression levels between the different time points (Fig. 10, A, middle column). In fact, DSas-4-GFP protein expression was higher at 1 mM of copper, reaching its maximum expression at 72h of induction (Fig. 10, A, left-hand column). Importantly, the western blot analysis also showed that control transfected cells that were not induced expressed basal levels of DSas-4-GFP protein (Fig. 10, A, without induction). Yet, the variability intrinsic to each experiment should be taken into account as repetition of the western blot in the previous conditions have also revealed that the basal DSas-4-GFP

pool from non-induced control transfected cells might not be present, as well as the expression levels of exogenous DSas-4-GFP protein can vary slightly between timepoints (but not cooper concentration) from different cell extracts (data not shown). In spite of the variability of the protein expression associated with each timepoint, the overall DSas-4-GFP expression levels were increased at 1 mM of copper concentration rather than at 0.5 mM in all western blot experiments.

After confirming the expression of the DSas4-GFP protein in the control stable cell line the following parameters were taken into account to determine the proper conditions for analysis and visualization of DSas-4-GFP: (1) population doubling time from Jupiter and *DSas-4 -/-* cell lines, (2) western blotting analysis of DSas-4-GFP expression and (3) the presence of Jupiter-GFP protein that marks for the MT array in both cell lines.

Relative to the first parameter, it was already shown by laser ablation experiments that *de novo* centriole formation in S-phase arrested vertebrate cycling cells (e.g. CHO) takes approximately 24h after centrosome ablation (Khodjakov et al., 2002), which is greater than the duration of a complete cell cycle (~18h for CHO cell line), suggesting that the kinetics for *de novo* centriole assembly in S-phase is slower than the normal cell cycle through the template mechanism. Thus, in order to avoid an analysis in which the timing for *de novo* centriole production would not be sufficient, the doubling population time was determined for control and acentriolar *DSas-4 -/-* cells (~36h for both cell lines, see material and methods). Regarding the expression profile of DSas4-GFP through western blot analysis the highest protein expression was observed with 1mM of $CuSO_4$. Finally, by immunofluorescence analysis it was possible to visualize the direct DSas4-GFP signal over the Jupiter-GFP, constitutively expressed in this cell line (Fig. 10, B and C).

It was previously shown that heterologous protein expression in *Drosophila* S2 cells increase progressively from lower (e.g. 24h) to high periods of induction (e.g. 72h) and fluorescence intensity is influenced by $CuSO_4$ concentration (Santos et al., 2007). In agreement, recombinant DSas-4-GFP signal was also detected in the alternative optimization conditions, such as 24h and 48h at 0.5 mM of copper concentration, but in the majority of cells the fluorescence intensity of DSas-4-GFP signal was too faint to be reliable for future experiments (data not shown).

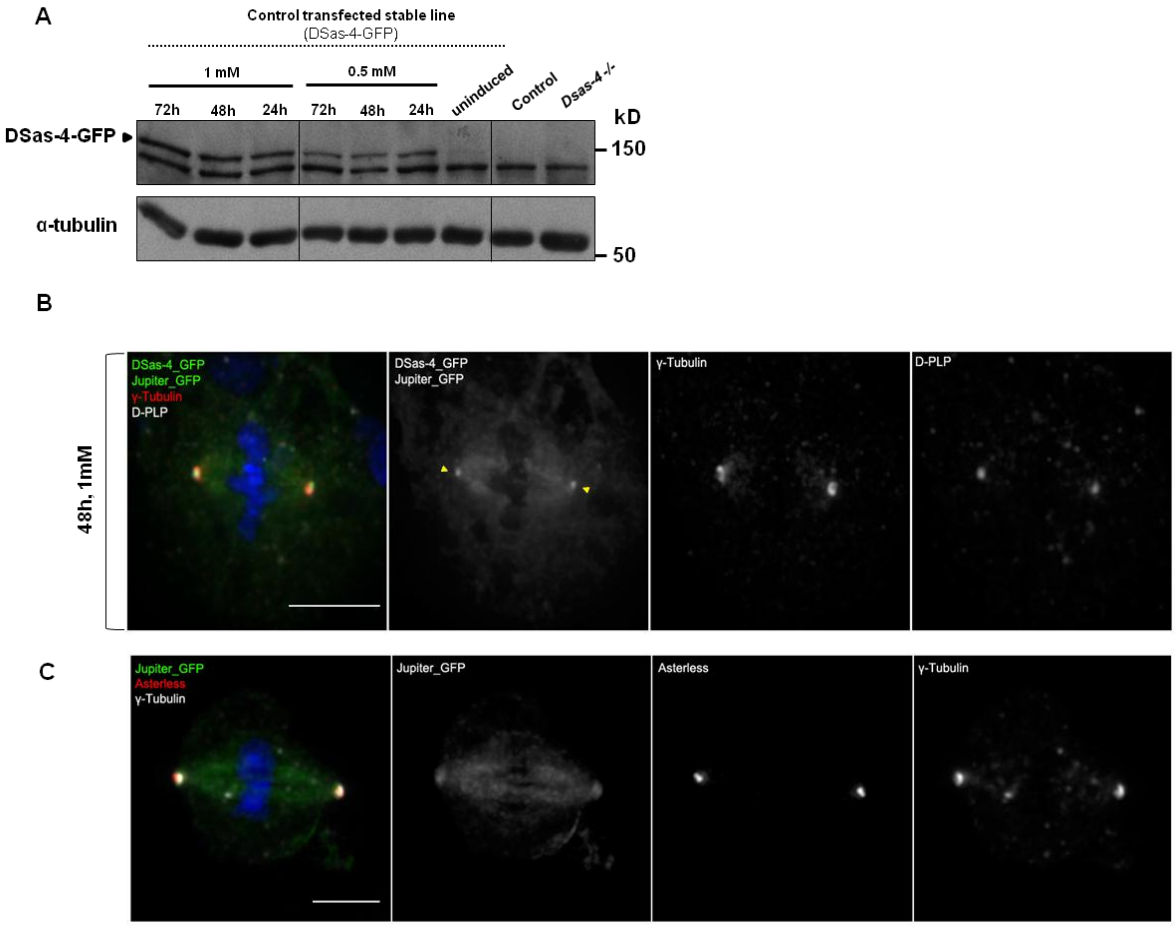


Figure 10. GFP fusion of DSas-4 protein is expressed and localizes to centrioles in stably transfected control Jupiter cells. (A) Immunoblotting of DSas-4-GFP expression levels according with $CuSO_4$ concentration and induction time in asynchronously control and *DSas-4 -/-* non-transfected cell lines, as well as control transfected stable cells. Lysates were prepared at the indicated time points and were analyzed using an anti-GFP antibody. α-tubulin was used as a loading control. DSas-4-GFP molecular weight: 130 KDa. **(B)** Fixed cell analysis in control Jupiter cells through direct detection of DSas-4-GFP fusion protein at the optimized parameters (48h, 1mM $CuSO_4$). The yellow arrowheads point to DSas-4-GFP signal at centrioles of a metaphase cell, which colocalizes with the centrosomal markers, γ-tubulin and D-PLP. Note that Jupiter-GFP is staining the mitotic spindle. Cells were stained for DNA (blue), γ-tubulin (red) and D-PLP (white). **(C)** Non-transfected control cell showing Jupiter-GFP signal staining to the MT array. Centrosomes were stained with Asl (red) and γ-tubulin (white). Scale bars in (B) and (C), 10 and 5 μm.

Taking all these factors into account, it was established that the condition to be used during the entire study for DSas-4-GFP visualization and analysis would be 48h with induction of 1mM of $CuSO_4$. These parameters allow the accomplishment of at least a first

cell cycle, as well as to yield enough DSas-4-GFP protein to guarantee its detection at centrioles over the strong Jupiter-GFP signal along the MT array.

Additionally, it was confirmed that using a higher concentration of $CuSO_4$ for the induction of the DSas-4-GFP protein did not affect cell viability neither disrupted DSas-4 localization, since it was possible to distinguish the direct DSas-4-GFP signal localizing to the centrioles throughout cell cycle (Fig. 10, compare B and C).

PART 3

PART 3.

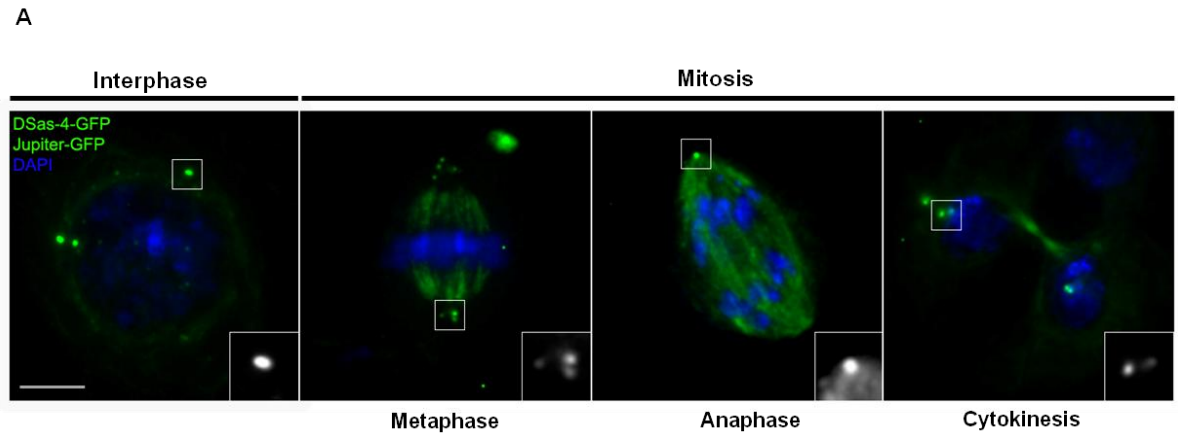
3. Analysis of *de novo* centriole formation in *DSas-4 -/-* rescued cells

3.1. Rescue of *DSas-4 -/-* cells through DSas-4 protein reintroduction

The goal of this study was to address *de novo* centriole biogenesis in somatic *DSas-4 -/-* mutant cells. Therefore, in order to rescue the DSas-4 mutant phenotype, the DSas-4-GFP construct was transfected in the *DSas-4 -/-* cell line background, and transfected cells were selected for the establishment of a stable cell line. The induction timing and copper concentration used for induction of expression of the recombinant DSas-4-GFP protein in transfected *DSas-4 -/-* cells was in accordance with the previously selected condition (1mM $CuSO_4$, 48 h), defined from the protein expression optimization carried out in control cells (Part 2, 2.1.2).

Figure 11 shows that the direct detection of DSas-4-GFP signal was successful using the established optimization condition. Transfected *DSas-4 -/-* rescued (*DSas-4 -/- R*) cells expressed the recombinant DSas-4 protein throughout the cell cycle, both in interphase and mitosis, in which DSas-4-GFP foci varied in size as well as in number (Fig. 11, A). Interestingly, during mitosis, the *de novo* DSas-4-GFP foci associated with the poles of the mitotic spindle (Fig. 11, A, metaphase cell).

The western blot analysis for the expression of DSas-4-GFP protein using an antibody raised against DSas-4 protein confirmed the successful establishment of a transfected *DSas-4 -/-* cell line expressing the exogenous DSas-4-GFP protein (Fig. 11, B, right-hand column) whereas, as expected, non-transfected acentriolar *DSas-4 -/-* cells exhibited the absence of DSas-4-GFP protein (Fig. 11, B, left-hand column).



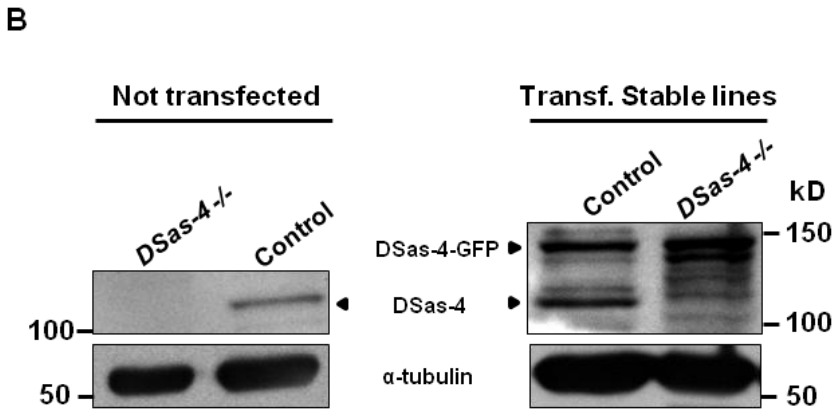


Figure 11. *DSas-4 -/-* rescued cells express DSas-4-GFP foci which associate with the poles of the mitotic spindle. (A) Immunofluorescence on fixed cells. *DSas-4 -/- R* cells were stained for DNA (blue). Note that both Jupiter-GFP and DSas-4-GFP signals were direct detected. From left to right: interphase; metaphase; anaphase; cytokinesis. Scale bar, 5 μm. Higher magnifications 3x. **(B)** Immunoblotting of DSas-4-GFP expression levels in asynchronous control and *DSas-4 -/-* non-transfected cell lines, as well as control and *DSas-4 -/-* transfected stable cells. Lysates were prepared at the optimized parameters (1 mM $CuSO_4$, 48 h), and were analyzed using an anti-DSas-4 antibody. α-tubulin was used as a loading control. DSas-4-GFP and DSas-4 molecular weight: 130 KDa and 103 KDa, respectively.

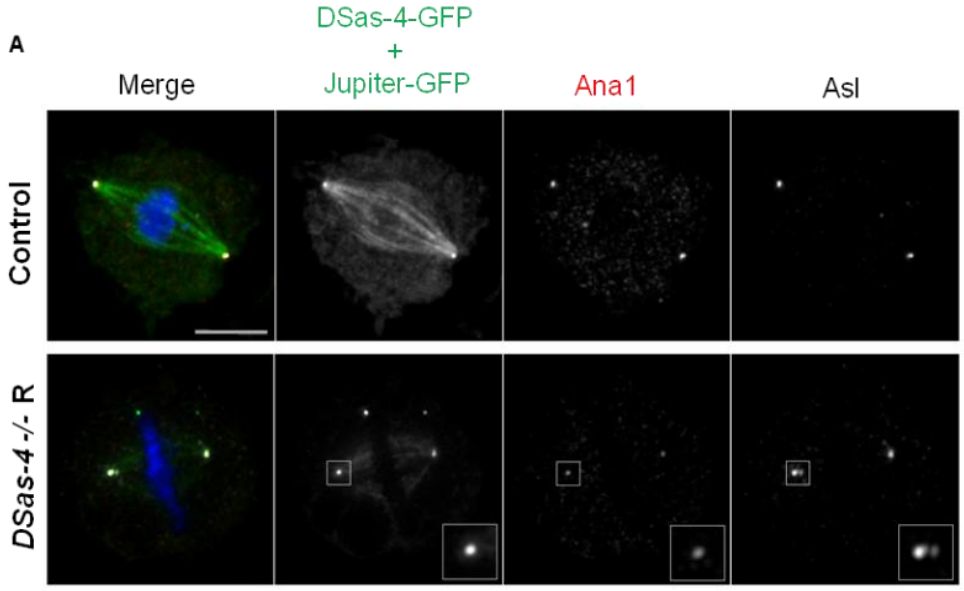
3.2. *de novo* formed DSas-4-GFP foci colocalize with centriolar proteins

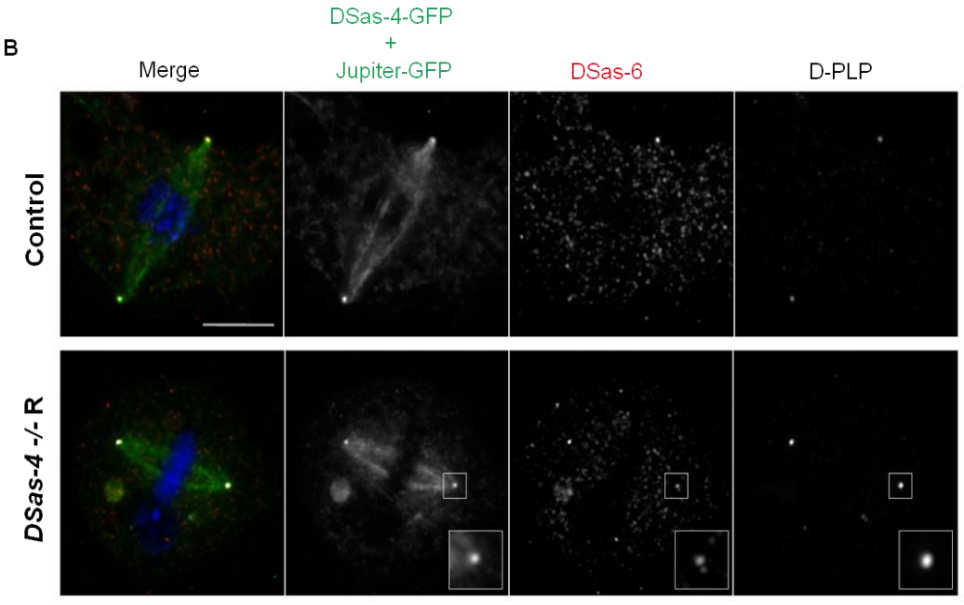
In order to verify the specificity of the produced DSas-4-GFP foci in the transfected *DSas-4 -/-* cell line, an immunofluorescence analysis was performed using specific centriolar markers, such as Ana1, DSas-6, Asl and D-PLP. As expected, mitotic control cells showed colocalization between DSas-4-GFP signal and the centriolar proteins Ana1, DSas-6, Asl, (Fig. 12A and B, upper panel), as well as D-PLP (Fig. 12B, upper panel).

Additionally, in the majority of the cases, the colocalization of DSas-4-GFP signal with the centriolar proteins Ana1, DSas-6, Asl and D-PLP (Fig. 12A and B, lower panel) was verified at the mitotic poles of *DSas-4 -/- R* cells.

Quantification of the colocalizations between DSas-4-GFP foci and the different centriolar markers revealed that 84 %, 73 % and 100 % of DSas-4-GFP foci colocalized with Ana1, DSas-6 and Asl in mitotic *DSas-4 -/- R* cells, respectively (Fig. 12A-D). Moreover, the centriole-wall associated D-PLP protein was found to be present in 100% of

DSas-4-GFP foci in mitotic *DSas-4 -/- R* cells, similarly to what was verified in control cells (96% of colocalizations in control cells) (Fig. 12B and C).





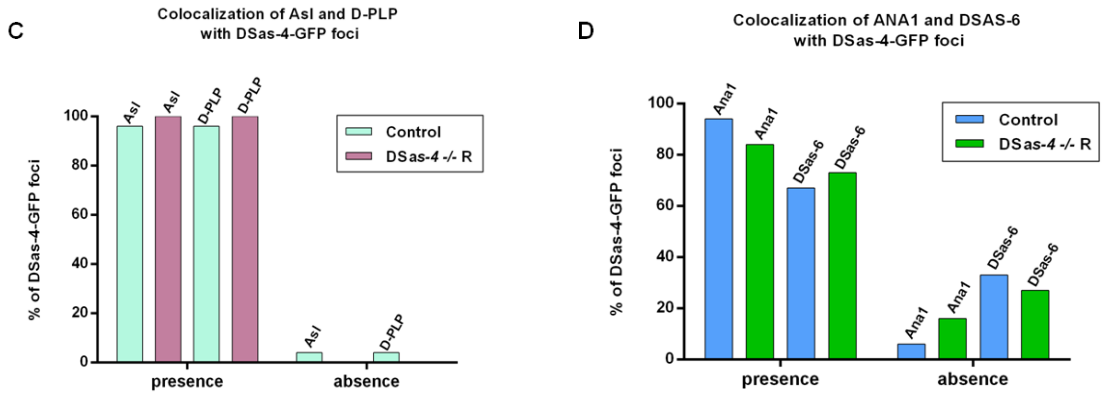


Figure 12. *de novo* formed DSas-4-GFP foci colocalize with Ana1, DSas-6, Asl and D-PLP in *DSas-4 -/- R* cells. (A) and **(B)** Immunofluorescence on fixed cells. Note that both Jupiter-GFP and DSas-4-GFP signals were directly detected. (A) Control and *DSas-4 -/- R* cells were stained for DNA (blue) and the centriolar markers Ana1 (red), Asl (white). (B) Control and *DSas-4 -/- R* cells were stained for DNA (blue) and the centriolar markers DSas-6 (red), D-PLP (white). DSas-4-GFP foci and respective colocalizations are shown at higher magnification. Scale bar, 5 μm. Higher magnifications 3x. **(C)** and **(D)** Quantification of the colocalizations between DSas-4-GFP foci and Ana1, DSas-6, Asl and D-PLP in metaphase cells. Blue, pink and green bars represent percentage of DSas-4-GFP foci and respective colocalizations in Jupiter transfected control cells and *DSas-4 -/- R* cells, respectively. Presence of colocalization for each antibody: DSas-6 (control (67%), *DSas-4 -/- R* (73%)); Ana1 (control (94%), *DSas-4 -/- R* (84%)); Asl (control (96%), *DSas-4 -/- R* (100%)); D-PLP (control (75%), *DSas-4 -/- R* (100%)). A total number of control (n=39, DSas-6; n=40, D-PLP; n=51, Ana1; n=35, Asl) and *DSas-4 -/- R* (n=16, DSas-6; n=17, D-PLP; n=31, Ana1; n=27, Asl) mitotic cells were analyzed from two or three independent experiments.

It is interesting to note that only 67 % control cells (in opposition with 73 % of *DSas-4 -/- R* cells) showed DSas-6 colocalization with DSas-4-GFP foci. This unexpected result might have been the consequence of antibody acessibility problems. A similar explanation can be applied for the D-PLP and Asl staining, since 100% of *DSas4 -/- R* cells showed colocalization in comparison with 96% in control cells (Fig. 12C).

3.3. Amplification of DSas-4-GFP foci in *DSas-4 -/-* rescued cells

To test whether DSas-4-GFP reintroduction could drive the formation of extra DSas-4-GFP foci, a quantitative analysis by immunofluorescence using the previously discussed centriolar markers was performed.

It was verified that 54 % of *DSas-4 -/- R* cells exhibited more than two DSas-4-GFP foci *per* mitotic cell compared with only 14 % of control transfected cells (Fig. 13B). Furthermore, 67 % and 19 % of mitotic control cells showed two or less than two DSas-4-GFP foci, whereas only 29 % and 17 % of *DSas-4 -/- R* cells exhibited two or less than two DSas-4-GFP foci *per* mitotic cell (Fig. 13B).

It is worth noting that extra DSas-4-GFP foci were found frequently either clustered at both or one of the poles of the mitotic spindle (Fig. 13A, square box), or dispersed in the cytoplasm of *DSas-4 -/- R* mitotic cells (Fig. 13A, lower panel).

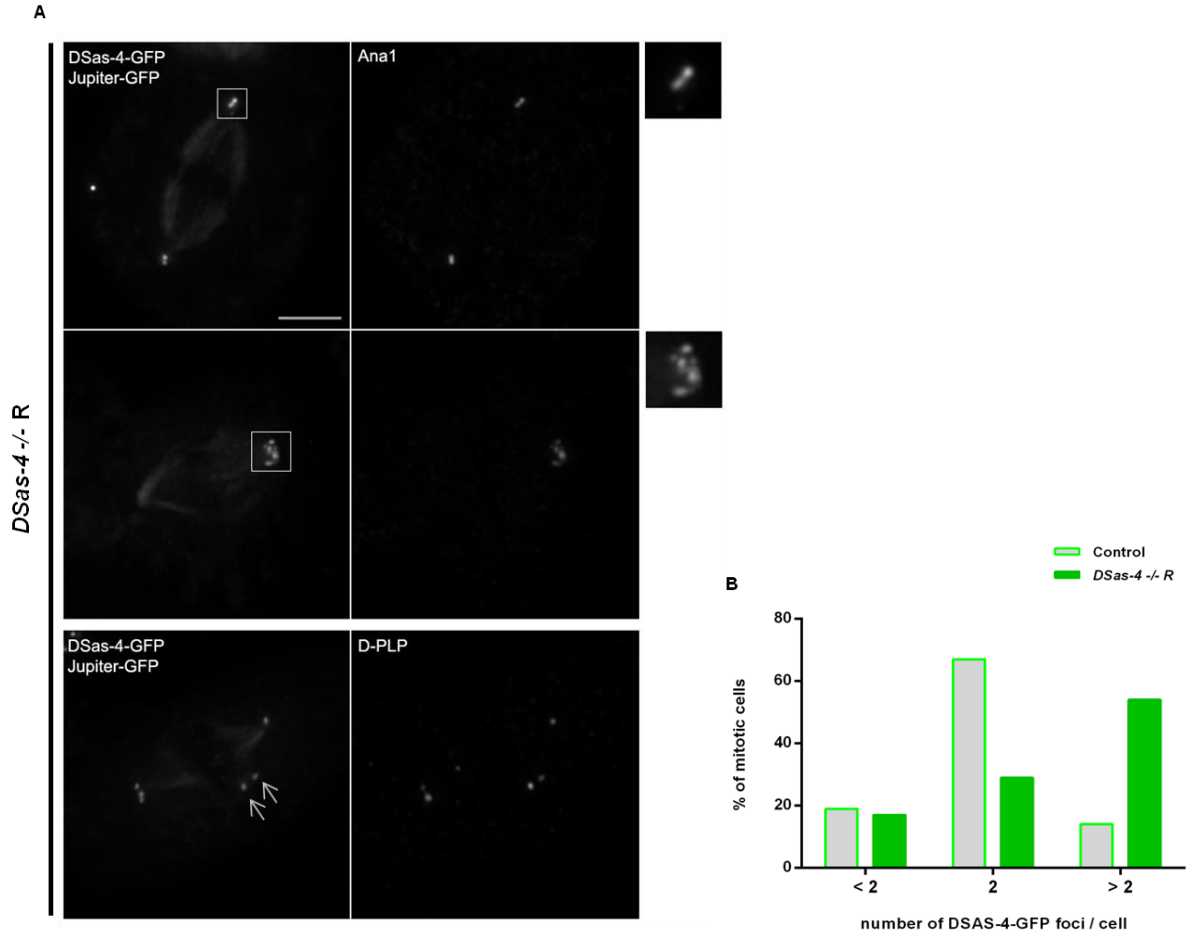


Figure 13. Induction of DSas-4-GFP expression in *DSas-4 -/-* rescued cells generates extra DSas-4-GFP foci. (A) Immunofluorescence on fixed cells. *DSas-4 -/- R* cells were stained for DNA (blue), Ana1 (red) and D-PLP (white). Note that both Jupiter-GFP and DSas-4-GFP signals were directly detected. Square boxes are magnifications of DSas-4-GFP foci aggregates/clustered at the mitotic poles and the arrows are pointing for DSas-4-GFP foci dispersed in the cytoplasm. Scale bar, 5 μm**.** Higher magnifications 3x. **(B)** Percentage of mitotic cells exhibiting less than two (<2), two or more than two (>2) DSas-4-GFP foci. Note that cells in which DSas-4-GFP foci did not

colocalize with centriolar markers were not analyzed. A total number of control (n=90) and *DSas-4 -/- R* (n=48) cells were analyzed from the stainings showed in figure 12.

3.4. Recovery of the expression levels of centriolar proteins in *DSas-4 -/-* rescued cells

To investigate a possible rescue in expression levels of specific centriolar and centrosomal proteins, a western blot analysis with *DSas-4 -/- R* cell extracts was performed using centriole and PCM-specific antibodies such as, Asl, DSas-6 and Cnn.

In fact, it was verified a rescue, in the *DSas-4 -/- R* cell line, of the expression levels of the centriolar proteins DSas-6 and Asl, but not of the PCM component Cnn (Fig. 14). Figure 14A shows that Asl protein expression levels dramatically increased, after rescue of DSas-4*,* comparing with the protein levels in the mutant *DSas-4 -/-* cell line, which were completely absent. Interestingly, a qualitative analysis suggests an increase in Asl expression levels in *DSas-4 -/- R* cells similar to control cells.

Furthermore, there was also an increase in the expression levels of the centriole structural protein DSas-6 in *DSas-4 -/- R* cells, although this increase was not as dramatic as with Asl (Fig. 14B).

In opposition, a positive rescue in protein levels was not verified for the PCM protein Cnn, since the expression levels of this protein seemed not to change dramatically from the rescued *DSas-4 -/-* cell line to the mutant *DSas-4 -/-* cells (Fig. 14C).

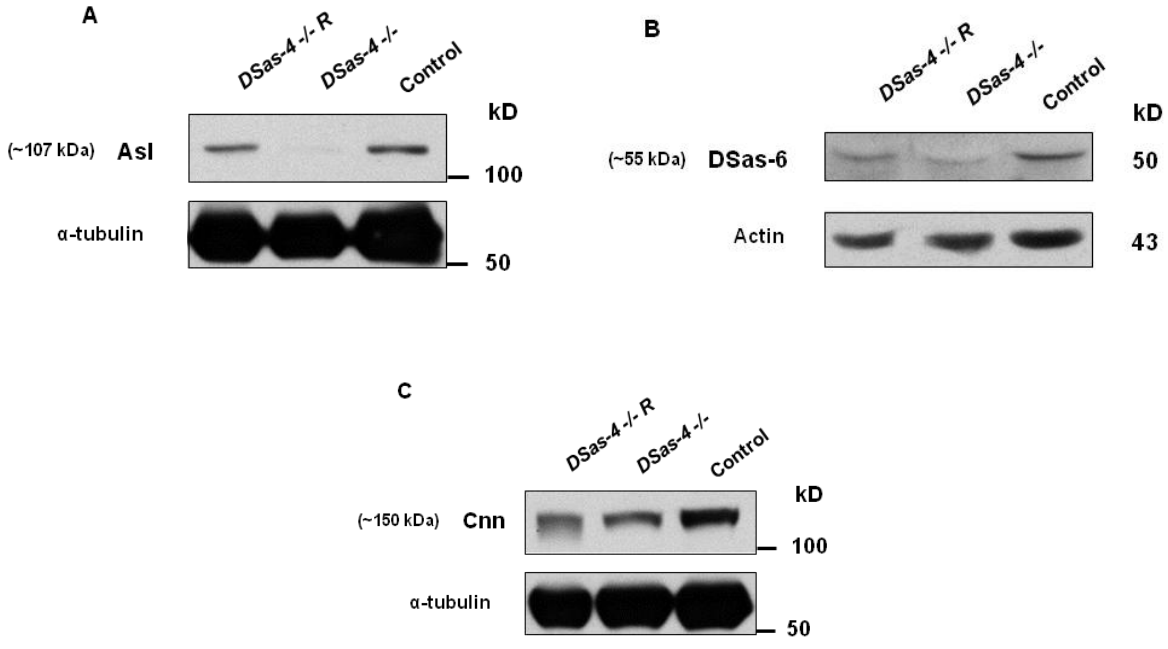


Figure 14. Rescue of *DSas-4 -/-* mutant cells led to the recovery of centriolar protein levels, but not of the PCM protein Centrosomin. (A), (B) and **(C)** Immunoblotting of protein expression levels of Asterless, DSas-6 and Cnn, respectively, in asynchronous Jupiter control non-transfected, *DSas-4 -/-* non-transfected and *DSas-4 -/-* rescued stable cell lines. Lysates were prepared at the optimized parameters (1 mM $CuSO_4$, 48 h), and were analyzed using an anti-Asl, DSas-6 and Cnn antibodies. α-tubulin (55 kDa) was used as a loading control for Asl and Cnn immunoblottings and actin(45 kDa) for DSas-6 immunobloting.

3.5. The *de novo* formed DSas-4-GFP foci are able to nucleate microtubules and to accumulate Centrosomin during mitosis

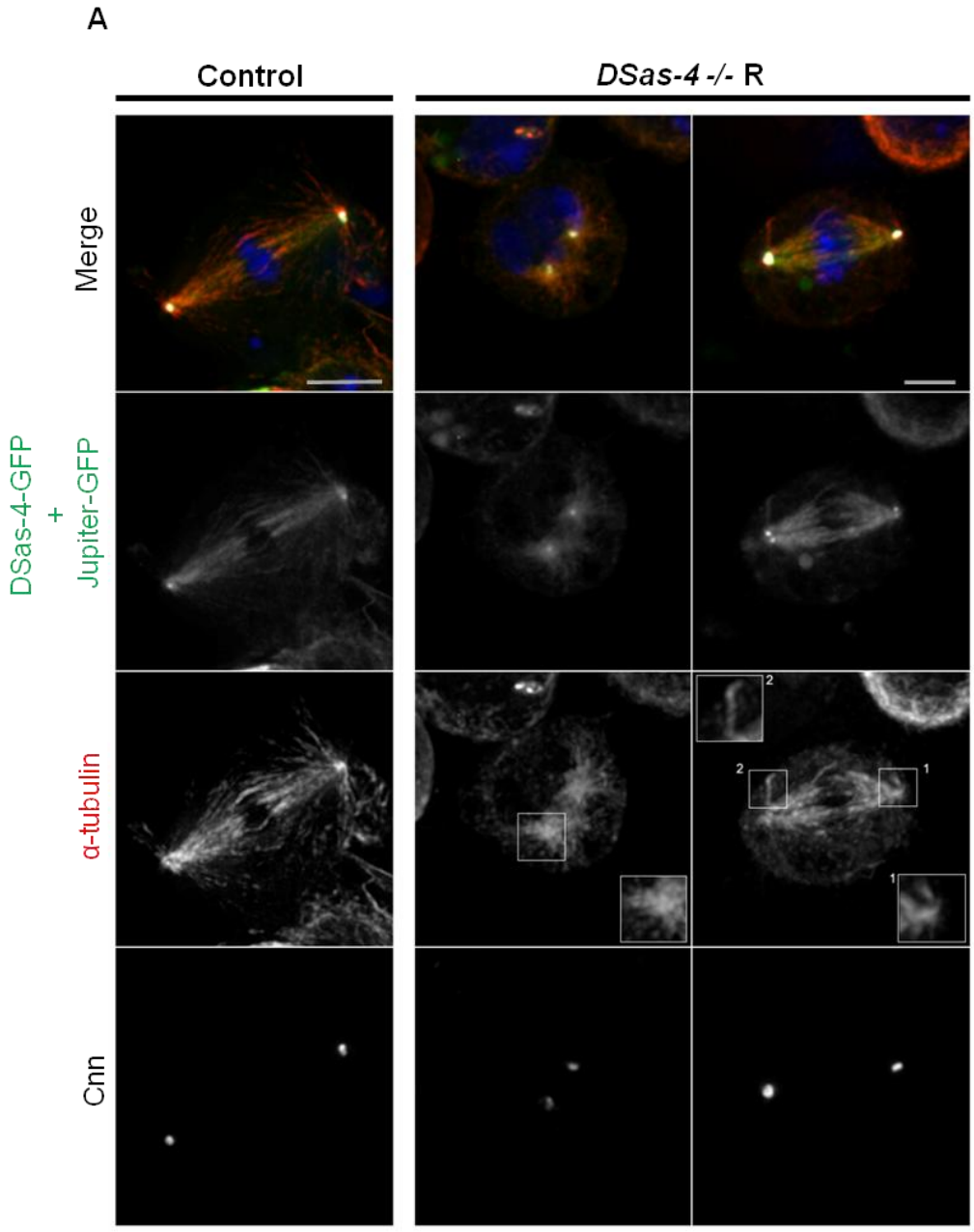
Centrioles organize the two opposite poles of the mitotic spindle through the production of astral microtubules during the first stages of mitosis (Meunier et al. 2012). Given the evidence for a specificity of *de novo* DSas-4-GFP foci for centriolar markers (see part 3, 3.2.), it was addressed if microtubule organizing capacity was restored in *DSas-4 -/- R* cells, and therefore, if DSas-4-GFP foci could potentially nucleate microtubules during mitosis.

Immunofluorescence analysis using α-tubulin to stain microtubules and the PCM protein Cnn suggests that DSas-4-GFP reintroduction led to the origin of microtubule fibers emanating from DSas-4-GFP foci in *DSas-4 -/- R* mitotic cells, although to a less extent than control cells (Fig. 15A, compare control and *DSas-4 -/- R*). The size and number of the produced microtubules was variable, as well as they were usually more discrete and in less number compared with the astral microtubules of control cells (Fig. 15A, see magnifications).

Moreover, Cnn colocalized with DSas-4-GFP foci and its recruitment and localization was more restricted to a single dot at both poles in *DSas-4 -/- R* cells, comparing with the presence of multiple Cnn aggregates in the non-transfected *DSas-4 -/-* cell line (see part 1, 1.2.) (Fig. 15, A, lower panel).

Given the previous evidence for MT nucleation capacity of DSas-4-GFP foci, it was investigated the relationship between the newly acquired microtubule capacity and mitotic spindle morphology in *DSas-4 -/-* R cells. Figure 15B shows that 83 % of control cells exhibit a polarized bipolar mitotic spindle (Fig. 15, A, left panel), and only 17 % present an undetermined spindle shape. In contrast, 50 % and 21 % of *DSas-4 -/- R* cells exhibited a WT-like and dual mitotic spindle, followed by 13 %, 2% and 15 % that showed a barrel, non-polarized and undetermined mitotic spindle morphology, respectively. Although the majority of rescued *DSas-4 -/-* cells exhibited a WT-like mitotic spindle, the overall

percentage of *DSas-4 -/- R* cells in each spindle morphology category did not significantly change in comparison with non-transfected *DSas-4 -/-* cells (compare Fig. 15B and Fig. 5B from part 1, 1.1.), in spite of the presence of astral microtubules in *DSas-4 -/- R* cell line.



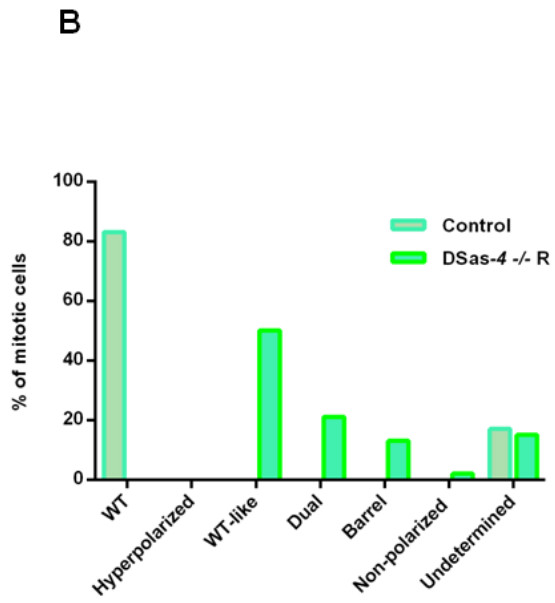


Figure 15. *de novo* formed DSas-4-GFP foci are able to nucleate microtubules. (A) Immunofluorescence on fixed cells. Control and *DSas-4 -/- R* cells were stained for DNA (blue), α-tubulin (green) and Cnn (white). Note that DSas-4-GFP signal was directly detected. Magnifications highlight astral microtubule fibers emanating from DSas-4-GFP foci at the mitotic poles. Scale bar, 5 μm. Higher magnifications 3x. **(B)** Percentage of mitotic cells *per* category of spindle morphology in control cells: WT (83%) and Undetermined (17%); *DSas-4 -/- R* cells: Barrel (13%); Dual (21%); WT-like (50%); Hyperpolarized (0%); Non-polarized (2%); Undetermined (15%). A total number of control (n=89) and *DSas-4 -/- R* (n=48) cells were analyzed from the stainings showed in figure 12.

In order to address in living cells the functionality of the reported DSas-4-GFP foci, live cell imaging was performed. The analysis showed that *DSas-4 -/- R* cells were able to produce *de novo* DSas-4-GFP foci in interphase, from which discrete astral microtubules were generated at the beginning of mitosis (Fig. 16, A and B). Interestingly, and in agreement with the fixed cell analysis, the live imaging data suggests that some *DSas-4 -/- R* cells were able to produce a more focused spindle (Fig. 16, A) whereas, in opposition to control cells, the mitotic spindle morphology in other *DSas-4 -/- R* dividing cells was non-focused, in spite of the microtubule generation capacity from the DSas-4-GFP foci (Fig. 16, B). Moreover, *DSas-4 -/- R* cells also showed more than two DSas-4-GFP foci in interphase and, consequently, the mitotic cells were multipolar (Fig. 16, B). Surprisingly, the duration of mitosis was different between cells generating different spindle morphologies. A qualitative analysis suggests that cells with non-focused spindles were slower in producing the mitotic spindle whereas *DSas-4 -/- R* cells with a more focused spindle completed mitosis in half of the time (compare fig. 16, A and B).

Nevertheless, the live cell imaging analysis to *DSas-4 -/- R* cells should be repeated for more conclusive results.

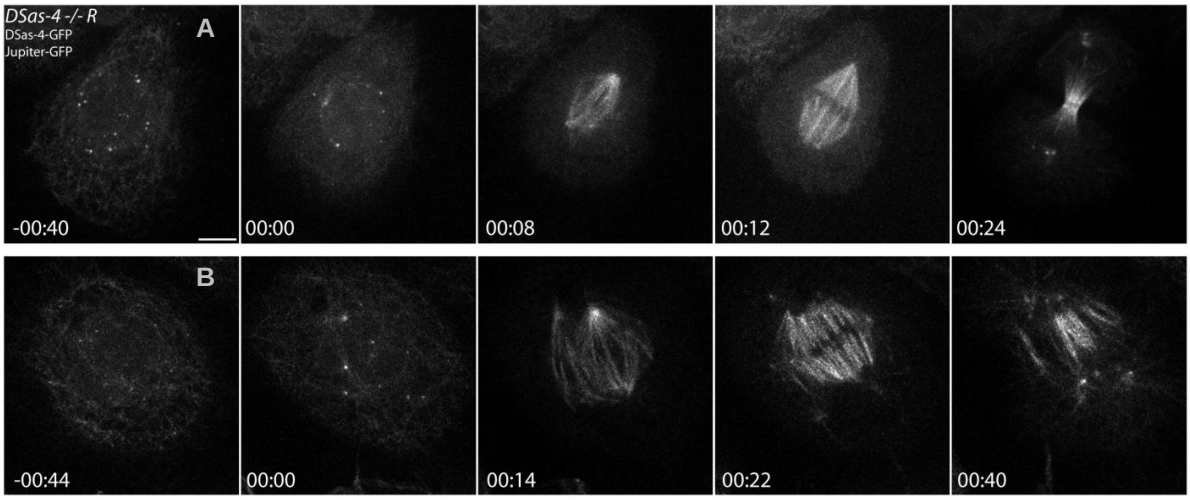


Figure 16. Live *DSas-4 -/-* rescued cells are able to generate astral MTs and produce a mitotic spindle from DSas-4-GFP foci. (A) and (B) live imaging of mitotic *DSas-4 -/-* cells expressing DSas-4-GFP and Jupiter-GFP. Cells seemed to progressively establish the mitotic spindle from discrete MTs bundles nucleated by DSas-4-GFP foci. The formed spindles might be nearly focused (A) or presenting a non-focused morphology (B). From left to right: interphase, prophase, metaphase and cytokinesis. The optimized parameters (1 mM $CuSO_4$, 48 h) were used for the induction of DSas-4-GFP protein expression. Scale bar, 5 μm. Time is in minutes. A total of six movies were analyzed.

3.6. *de novo* centriole-like structures are formed in *DSas-4 -/-* rescued cells

The results so far suggested that expression of DSas-4-GFP, in the mutant background, is able to induce the formation of foci, that even though are able to recruit other centriolar and centrosomal proteins, don't seem totally functional because spindle morphology is not fully recovered when compared to the DSas-4 mutant cells.

To better understand the ultra structure of these Dsas-4-GFP foci, an electron microscopy analysis was performed (collaboration with Alain Debec, IJM, France). The data revealed that centriole-like structures were formed *de novo* in *DSas-4 -/-* cells expressing DSas-4-GFP recombinant protein. A qualitative analysis showed that some of these structures resembled morphologically recognizable centrioles with the presence of the inner central hub and the outer microtubule wall (Fig. 17, square boxes in A and B).

Strikingly, in one cell it was possible to observe a centriole-like structure surrounded by microtubule fibers (Fig. 17A, arrows).

The EM analysis also revealed the presence of electron-dense material clouds that did not contain centriole-like structures (Fig. 17D).

Nevertheless, electron microscopy analysis should be repeated in order to confirm and better characterize the *de novo* formed centriole-like structures in *DSas-4- -/- R* cells.

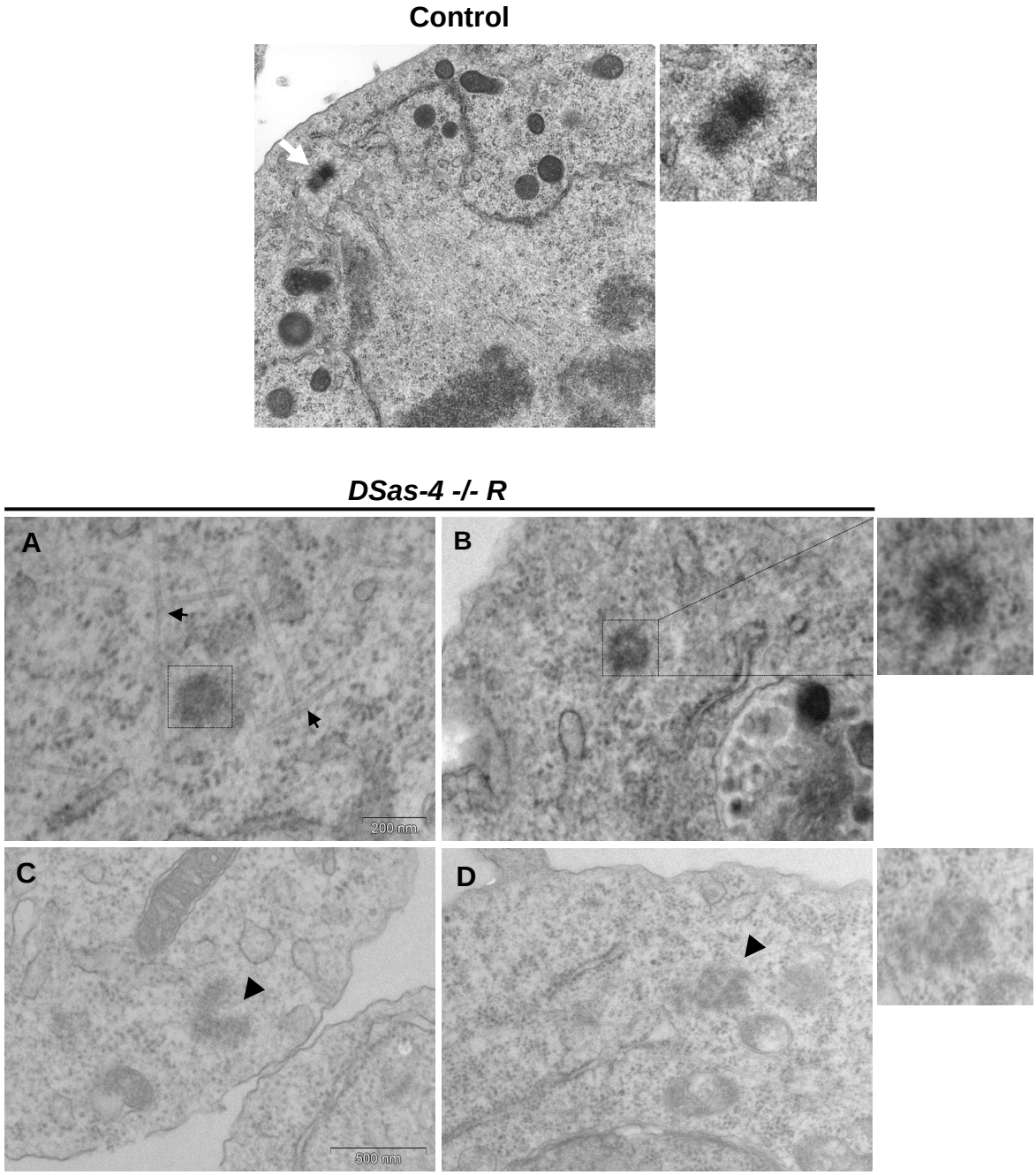


Figure 17. Centriole-like structures are formed in *DSas-4 -/- R* cells. Control non-transfected mitotic cell and *DSas-4 -/- R* cells. White arrow in control cell represents a canonical centrosome. **(A)** and **(B)** are examples of *de nov*o formed centrioles that seem to be structurally normal in *DSas-4 -/- R* cells (square box). Arrows represent microtubule fibers that seem to emanate from the centriole-like structure. **(C)** and **(D)** arrowheads represent the presence of electron-dense material clouds without centriole-like structures. The optimized parameters (1 mM $CuSO_4$, 48 h) were used for the induction of DSas-4-GFP protein in *DSas-4 -/- R* cells. Lateral images are higher magnifications of B and D, 4x. Scale bar, (B), (C) and (D), 500 nm, (A), 200nm.

4. DISCUSSION

DSas-4 -/- cells show abnormal spindle morphology

The heterogeneity regarding the mitotic spindle modalities in *DSas-4 -/-* mutant cells likely reflects the acentriolar nature of this cell line. The wild-type cells showed well polarized bipolar mitotic spindles with many astral MTs irradiating from each centrosome, which function as the main MT assembly centers for mitotic spindle formation during mitosis. The acentriolar spindles of *DSas-4 -/-* cells did not show, for any of the morphological type, astral MTs. The absence of astral MTs at the poles is perhaps the main proof of the lack of centrioles, which is consistent with the absence of a functional centrosome able to nucleate MTs. Yet, since centrosomes are dispensable in cell division (Debec et al., 2010), acentriolar *DSas-4 -/-* cells build their mitotic spindle in a "inside-outside" process, which means that MTs start to be nucleated from several foci close to chromatin. From this study, it is evident the existence of five acentriolar mitotic spindle types that exhibited a polarized “gradient”, from more focused (barrel-shaped, dual, hyperpolarized and WT-like) to non-polarized spindles. Interestingly, the gradient in the polarization associated with a variety of morphological acentriolar spindles was also reported in previous studies in *Drosophila* and HeLa acentriolar cells (Bobinnec et al. 1998; Debec et al. 1995).

According with Lecland et al. (2013), the most frequent acentriolar spindle type was the barrel-shaped (29%) followed by dual (25%) spindle, and the less frequent were the hyper- (15%) and non-polarized (17%) spindles. In this study, however, it was found that the WT-like was the most predominant spindle over the barrel-shaped. This difference might come from the subjective component implicit in the categorization of spindle shape. Nevertheless, in spite of the divergence between the frequencies of mitotic spindles in both studies, it is clear that the three major anastral spindle categories are the WT-like, barrel-shaped and the dual, and the less frequent are the non- and hyper-polarized spindles (in agreement with Lecland et al. 2013). It is also possible to conclude that, in spite of the absence of centrioles, and therefore of a proper centrosome, *DSas-4 -/-* cells are still able to produce more frequently focused spindles rather than non-polarized spindles in mitosis. Together these data demonstrate that, although centrosome function is compromised due to the lack of centrioles, cells are able to overpass that deficit and successfully undergo mitosis through the assembly of an anastral mitotic spindle via a centrosome independent pathway.

Acentriolar *DSas-4 -/-* cells recruit PCM and centriolar components to the poles of the mitotic spindle

The immunofluorescence analysis of the acentriolar *DSas-4-/-* cells revealed that the PCM components Cnn and γ-tubulin, as well as the centriolar proteins D-PLP and Asl can be recruited to the poles of mitotic acentriolar cells. The presence of γ-tubulin and Cnn in mitotic *DSas-4 -/-* cells in this study is in stark contrast with the reported results by Lecland et al. (2013), in which their recruitment or presence at the mitotic poles was not observed.

Interestingly, the recruitment of γ-tubulin to the poles of an acentriolar mutant *Drosophila* cell line (1182-4 cell line) was previously described to occur. It was shown that 70% of the mitotic acentriolar cells exhibited a faint presence of γ-tubulin at the spindle extremities, in which small dispersed granules could also be present. Moreover, 30% of metaphase cells showed a small fluorescent staining pattern reminiscent of those observed in wild type cells. These more similar WT-spots of γ-tubulin at the extremities of MT bundles in 1182-4 mitotic cells were shown to be related with more polarized anastral spindles. Nevertheless, microtubule disassembly assays demonstrated the absence of a proper centrosome through the disappearance of the γ-tubulin focus and the absence of astral microtubules, from which the mitotic spindle starts to be formed and organized in wild type cells. Thus, it was concluded that the presence of γ-tubulin at the acentriolar mitotic spindle extremities is not equivalent to the presence of a true centrosome (Debec et al., 1995). In agreement with the reported γ-tubulin localization in the *Drosophila* 1182-4 cell line, this study confirms the presence of γ-tubulin at the acentriolar spindle extremities and it also demonstrates that the same type of localization can occur for the PCM and centriolar components: Cnn, D-PLP and Asl. In all three cases, the immunofluorescence analysis revealed that either small diffuse granules or more focused spots could localize to the extremities of microtubule bundles in *DSas-4 -/-* mitotic cells. In line with this spindle pole localizing PCM foci in *DSas-4 -/-* cells, the accumulation of PCM material in acentrosomal cells was also reported when centrioles were disintegrated through the injection of an antiglutamylated antibody into HeLa cells originating transient acentriolar cells. The immunofluorescence and electron microscopy analysis revealed that these cells also showed an unfocused organization of PCM components (e.g. γ-tubulin) at the acentriolar mitotic poles that appeared to be segregated in variable amounts to each pole (Bobinnec et al. 1998).

A recent characterization of the acentriolar spindle poles in 1182-4D cells performed by Moutinho-Pereira et al. (2009) have also shown the presence of PCM and centriolar proteins, such as Cnn, γ-tubulin, D-PLP and DSas-4. Nevertheless, and in

agreement with the previous data, all proteins exhibited a variable localization at the acentriolar spindle poles, ranging from well-defined foci to small aggregates highly dispersed in the spindle poles. Furthermore, it was also shown by live cell imaging that γ-tubulin aggregates concentrated at acentriolar spindle poles containing focused MT minus ends, therefore suggesting a relationship between the presence of γ-tubulin foci in more polarized acentriolar mitotic spindles.

Moreover, Joo-Hee Sir and co-workers (2013) recently established an acentriolar vertebrate DT40 cell line through disruption of the centriolar CEP152 and STIL genes. Strikingly, CEP152-KO and STIL-KO cells exhibited acentriolar MTOCs (aMTOCs) enriched in PCM components CDK5RAP2 and γ-tubulin, but in which both proteins lost the concentric shape characteristic of an ordered PCM. Since these acentriolar cell lines are DNA repair efficient, and it is known that many regulatory components linked to cell cycle-specific functions as well as DNA damage response lie at the centrosome, the authors suggested that the PCM foci described in acentriolar DT40 cells might perform a limited role in signaling. Thus, the PCM aggregates found in *DSas-4 -/-* mutant cells might also be partially involved in signaling.

The colocalizations between these PCM and centriolar markers revealed the potential formation of PCM aggregates or foci at the mitotic poles of acentriolar cells. In fact, it is known that Cnn, Asl and D-PLP can interact and form S-CAP complexes with SAS-4 protein (Conduit et al. 2010; Gopalakrishnan et al. 2011). Nevertheless, it is not possible to exclude that in the absence of SAS-4, these proteins can still interact and form complexes together that localize to the polar extremities of acentriolar cells.

The measurement of the fluorescence intensity levels for each protein showed a reduction (~70-90%) in their recruitment to the poles compared to control cells. This failure in the recruitment of Cnn, D-PLP and Asl is in accordance with the role of the centrioles in PCM formation. Indeed, it is known that centrioles greatly influence PCM stability, formation and size since their disintegration led to the scattering of the associated pericentriolar material (Bobinnec et al. 1998) and variations in centriole size led to variations in PCM size in *C. elegans* embryos (Kirkham et al. 2003). In *Drosophila*, it was also shown that mature centrioles regulate centrosome size through the incorporation of Cnn closest to the centriole vicinity, which seems to be driven by Asl and the PCM protein SPD-2. The presence of Cnn in the periphery of centrioles allows the recruitment of other centrosomal components like γ-tubulin and D-TACC, promoting centrosome maturation (Conduit et al. 2010). In the absence of centrioles, there is the lack of a platform that allows the centrosome to grow and consequently Cnn incorporation is significantly reduced.

Strikingly, a staining over the mitotic spindle was also verified for all markers. Given that Cnn, D-PLP and Asl were only reported to localize to centrosomes, the presence of these components to the microtubule array possibly represents their delocalization from the spindle poles.

In agreement with the relationship of a strong polar localization of γ-tubulin in more focused acentriolar mitotic spindles reported by Debec et al. (1995), it was shown that Asl presence at the acentriolar poles is related with the polarization of the mitotic spindle whereby is frequently present in WT-like and hyperpolarized spindles of *DSas-4 -/-* cells. Although the respective quantifications were not performed, this pattern of occurrence was also noticed for Cnn and D-PLP. Since in *DSas-4 -/-* cells, the mitotic spindle is formed through the chromatin pathway in an inside-outside process (Lecland et al., 2013), it is tempting to speculate that these PCM aggregates might act as cues for the polarization of the mitotic spindle, hence promoting the formation of a more WT-like spindle and ensuring fidelity in chromosome segregation.

The accumulation of the centriolar proteins D-PLP and Asl could suggest that mutant *DSas-4 -/-* cells could have MTOCs probably containing structures resembling centrioles, such as disrupted centriolar walls, or nascent procentrioles due to the absence of SAS-4, questioning the total absence of the centriole structure in this cell line. In flies, D-PLP is known to be mostly associated with centrioles having an important role in PCM recruitment (Martinez-Campos et al. 2004; Menella et al. 2012) and Asl is a key player in centriole duplication in humans and flies (Dzhindzhev et al. 2010; Hatch et al. 2010). 3D-SIM microscopy revealed that both components are present in a ring-like structure localizing to the centriole wall (Fu et al. 2012; Sonnen et al. 2012), and specifically D-PLP is also present through the PCM (Menella et al. 2012; Steffen et al. 2012). Since D-PLP is also known to have a pool that specifically associates with the pericentriolar material and is not involved in centriole biogenesis process (Martinez-Campos et al. 2004), D-PLP foci in *DSas-4 -/-* acentriolar poles mostly represents the PCM associated pool of D-PLP. Asterless is an upstream component of centriole biogenesis cascade, but its function has been also linked to PCM recruitment (Blachon et al. 2008; Conduit et al. 2010, Varmark et al. 2007). Thus, concomitantly with D-PLP localization in acentriolar cells, the Asl foci at the poles of DSas-4 mutant cells probably reflects its role in PCM formation and not the presence of a true centrosome.

The acentriolar nature of *DSas-4 -/-* cell line was confirmed by electron microscopy analysis (Lecland et al. 2013). Moreover the absence of astral microtubules, the mode of microtubule assembly during mitosis and the absence of the centriolar specific proteins DSas-6 and Ana1 (that have only been related with centrioles and not PCM) (Blachon et al. 2009; Gopalakrishnan et al. 2011; Goshima et al. 2007; Rodrigues-Martins et al.

2007b; Stevens et al. 2010a) reported in this study supports the total absence of centrioles in this *Drosophila* cell line.

Ectopic DSas-4 protein rescues *DSas-4 -/-* acentriolar phenotype

Drosophila melanogaster cellular lines (e.g. S2 cells) have been, in the past years, intensively and successfully used as a system for the expression of heterologous proteins. The high transfection efficiency coupled to the ability to generate a stable polyclonal cell line expressing a constitutively or inducible biologically active recombinant protein have made this system as one of the most used in the cellular and molecular biology fields.

Reporter proteins such as mRFP (monomeric red fluorescent protein) have been used to track protein expression and localization. Nevertheless, it is well-known that the improvement in the expression of exogenous and recombinant proteins in the host system is a central point of study for high and proper gene expression.

In this work, DSas-4-mRFP plasmid was transfected circular and no previous digestion to linearize it was performed. Consequently, as incorporation of the plasmid into the cell genome is dependent on DNA linearization, there is a possibility that the cut in the construct was done in the middle of recombinant DSas-4 protein thereby affecting and interfering with its expression and function. Second, the protein expression analysis by western blot in order to confirm the successful expression of DSas-4-mRFP protein in stably transfected cells was not performed. Third, the DSas-4-mRFP plasmid was also not sequenced with the aim to confirm the nucleotide sequence of the construct. Finally, it is also not possible to exclude the differences between *Drosophila* cell lines physiology. Whereas most scientific works used Schneider 2 cells as expression system, here two different *Drosophila* cell lines were used, namely Jupiter and *DSas-4 -/-* cells. Although the three cell lines (S2, Jupiter and *DSas-4 -/-*) derived from *Drosophila* embryos, it is possible that Jupiter and DSas-4 mutant cells are more sensitive and thus are not so prone to transfections and exogenous protein expression.

Despite of the aforementioned limitations that could impair heterologous protein expression, there are some conditions performed in this study that would favor the expression of DSas-4-mRFP recombinant protein in control and *DSas-4 -/-* cells. Usually, stable transfected cell lines for the large scale of protein expression are generated through co-transfection, which means that both expression and selection plasmids are transfected independently of each other. Therefore, there is a tendency for a decrease in the protein expression levels during long culture periods by stable transformants due to the formation of a heterogeneous population, in which some cells have incorporated only

the plasmid with the selected marker without the gene of interest in the DNA. Importantly, these drug-resistant pool of cells have the ability to grow faster than the cells with the gene of interest plus the selection marker (Iwaki and Castellino, 2008). In order to overcome this problem, the resistance gene coding for Blasticidin S was cloned into the DSas-4-mRFP plasmid with the aim to increase the efficiency of transfection as well as the stability of the recombinant DSas-4 protein over long culture periods.

Moreover, since the induction of DSas-4-mRFP is under the metallothionein promoter (pMT), the protocol used in this study for the DSas-4-mRFP expression optimization would allow, in principle, to define the proper conditions for the expression of the fusion DSas-4-mRFP protein. The protocol was based on the copper sulfate (CuSO4) concentration and the duration of which DSas-4-mRFP expression was promoted. Santos and co-workers (2007) in an attempt to define the proper parameters for the expression of heterologous proteins under the metallothionein promoter in S2 cells, have demonstrated that increased recombinant protein synthesis is directly proportional to an increase in copper concentration as well as in induction time. In fact, it was verified that between 500 μM and 700 μM (0.5 and 0.7 mM, respectively) of copper sulfate concentration, protein expression was higher at 700 μM after three days (or 72 h) of induction (80% of cells compared with 60% of cells at 500 μM). Interestingly, there was a progressive increase in the fluorescence intensity of the recombinant protein after five days (or 120 h) of induction, in which stable transfected S2 cells showed an heterogeneous expression among the cell population. Furthermore, cell growth was not affected, even at copper concentration of 700 μM. Therefore, at least using one of those conditions (e.g. 500 μM at 72 h) it would be expected the expression and detection at centrioles of the fusion DSas-4-mRFP protein in control (Jupiter) and DSas-4 mutant cells. However, among all the copper concentrations tested (0.5 and 1 mM) and induction times (24, 48, 72, 96 h), there was almost absence of DSas-4-mRFP signal at centrioles in all stages of the cell, even when an anti-mRFP antibody was used.

It is also possible that mRFP-tagging of DSas-4 could have disrupted its centriolar localization, promoting its localization in other cell components. This effect was already reported for centriolar proteins tagged with GFP (green fluorescent protein) that localized to the plasma membrane, nucleus and nuclear envelope in *Drosophila* cells (Dobbelaere et al. 2008), although the authors also claimed the possibility of a more indirect role of those proteins in centriole duplication. Protein delocalization from centrioles seems unlikely to have happened in the stable transfected cell lines used in this study, since DSas-4-mRFP expression was never detected or associated with other cell compartments such as plasma membrane or nucleus.

The monomeric red fluorescent mCherry protein is highly photostable and thus more resistant to photobleaching in comparison with mRFP protein (Shaner et al., 2005). However, the establishment of stable transfected cell lines (Jupiter and *DSas-4 -/-*) using a single plasmid carrying the fusion protein DSas-4-mCherry as well as the resistance selection marker Blasticidin S also revealed similar results in terms of protein detection.

The results from the protein expression optimization carried out for the DSas-4-mRFP fusion protein in control cells are in stark contrast with the successful direct detection of DSas-4-mRFP in S2 cells. The last case suggests that, although no plasmid sequence or western blotting analysis was performed in this study, the unsuccessful detection of DSas-4-mRFP (or mCherry) signals at centrioles of the Jupiter control cell line was probably not due to a defect in the nucleotide sequence of the plasmid or to a delocalization from centrioles of the recombinant protein, and it might be more related with the differences between cell lines in response to heterologous protein expression or due to protein folding and stability problems.

Other alternative protocols that might have been used to more efficiently establish transfected stable cell lines in this study could have been the selection of transfected cells trough FACS (Fluorescence activated cell sorting) or cloning of a single transfected cell in order to originate a stable transfected cell line derived from a single cell, in which there is a more homogeneous recombinant protein expression and weeds out the possibility of growing drug-resistant cells.

de novo formed DSas-4-GFP foci colocalize with centriolar proteins

The successful establishment of a *DSas-4 -/-* cell line stably expressing DSas-4-GFP recombinant protein throughout the cell cycle allowed us to address the specificity of DSas-4-GFP foci using centriolar markers. In this study, the evidence of colocalization in the majority of *DSas-4 -/- R* cells between DSas-4-GFP foci and D-PLP, DSas-6, Asl and Ana1 at the mitotic poles suggests that DSas-4-GFP foci may not be just non-functional protein aggregates (Peel et al., 2007; Tang et al., 2009) due to the induction of DSas-4-GFP expression using the metallothionin promoter (pMT), (which does not allow a controlled protein expression) but rather, DSas-4-GFP foci may represent *de novo* centriole-like structures in dividing and rescued *DSas-4 -/-* cells. Furthermore, the previous proteins used to address DSas-4-GFP foci specificity have been used as a centriolar markers by several studies (Blachon et al., 2009; Dzhindzhev et al., 2010; Gopalakrishnan et al., 2012; Gopalakrishnan et al., 2013; Martinez-Campos et al., 2004; Peel et al., 2007; Rodrigues-Martins et al., 2007; Stevens et al., 2010).

In the last years, the *de novo* formation of centrioles has been adressed through different experimental approaches. From these studies, it is important to emphasize two studies in which *de novo* centriole-like structures were formed after overexpressing specific centriolar proteins in unfertilized *Drosophila* eggs. Rodrigues-Martins and co-workers (2007) have shown that SAK-overexpression in unfertilized eggs led to the formation of *de novo* centriole structures containing centriolar and PCM components such as DSas-6, γ-tubulin and Cnn. Interestingly, SAK-induced centriole biogenesis in acentriolar *Drosophila* S2 cells was dependent on the presence of the structural proteins DSas-4 and DSas-6. Moreover, Peel et al. (2007) have also shown that overexpression of DSas-6, SAK and specifically of DSas-4 induced the *de novo* formation of centriole-like structures in unfertilized *Drosophila* eggs that colocalized with specific markers such as D-PLP, Cnn and γ-tubulin. In agreement with the former study, it was also shown that extra centriole formation in larval brain cells through SAK-overexpression was also dependent on DSas-4 presence (Peel et al., 2007).

The role of DSas-4 in centrosome biology has been thoroughly studied. In spite of its essential function in the centriole duplication process, it was shown that DSas-4 is a very important player in PCM recruitment. It promotes the assembly of cytoplasmic complexes (S-CAP) composed by proteins such as Cnn, D-PLP and Asl (Gopalakrishnan et al., 2012; Gopalakrishnan et al., 2013). This is complemented with the fact that its overexpression can originate acentriolar MTOCs (Dzhindzhev et al., 2010; Gopalakrishnan et al., 2013). Thus, it might be that the produced DSas-4-GFP foci containing D-PLP and Asl could merely be S-CAP not containing native centrioles. There is also some evidence that *de novo* centriole assembly is preceded by the formation of extensive clouds of electron-dense material that lacks centrioles, but contain PCM components such as γ-tubulin (Khodjakov et al., 2002; La Terra et al., 2005). Thus, along with the DSas-4-dependent formation of S-CAP, it is possible that DSas-4-GFP foci staining for centriolar markers, mainly D-PLP and Asl, may represent electron-dense material lacking centrioles.

Nevertheless, and given the present data, there are several factors which favor the hypothesis that the *de novo* formed DSas-4-GFP foci in *DSas-4 -/- R* cells represent true centriole-like structures. First, the high percentage of colocalizations between centriolar markers and DSas-4-GFP foci in rescued *DSas-4 -/-* cells is indicative of DSas-4-GFP centriolar specificity. Second, the presence of DSas-6, a key protein in cartwheel assembly, points out the potential existence of a centriolar structure. Moreover, even though the protein fluorescence intensity levels for D-PLP, DSas-6, Ana1 and Asl were not measured in the *DSas-4 -/- R* cells (as it was performed for D-PLP and Asl in the mutant *DSas-4 -/-* cell line), the focused localization to a single dot of these centriolar

proteins at the mitotic poles of the majority *DSas-4 -/- R* cells is in contrast with the scattered localization or even absence verified for these markers in the mutant *DSas-4 -/-* cells. Thus, there is some evidence for a rescue of the acentriolar nature of *DSas-4 -/-* mutant cells after DSas-4-GFP expression, which supports the template-free mechanism in centriole biogenesis.

It is worth noting that this quantitative analysis by immunofluorescence using centriolar markers should be repeated since the total number of mitotic *DSas-4-/- R* cells analysed *per* condition was not enough to perform a statistical analysis.

Amplification of DSas-4-GFP foci in *DSas-4 -/-* rescued cells

Centriole amplification has been directly linked to the *de novo* centriole formation in all studies that have adressed this mode of centriole assembly. Either by centrosome laser ablation or through the overexpression of centriole-specific proteins, it was shown the expression of extra centrioles in *Drosophila* and in mammalian cells (A. Rodrigues-Martins et al., 2007b; Khodjakov et al., 2002; La Terra et al., 2005; Peel et al., 2007; Uetake et al., 2007).

In agreement with the previous studies, here it was shown that *DSas-4 -/- R* cells also formed more than two DSas-4-GFP foci *per* mitotic cell (54% of *DSas-4 -/- R cells*), whereas the majority of control mitotic cells only presented two foci (67%). Moreover, the localization of these foci in *DSas-4 -/- R* cells was variable. In some cells DSas-4-GFP foci were specifically localized in clusters at the mitotic poles or appeared randomly dispersed in the cytoplasm.

In support of these results, Rodrigues-Martins et al. (2007) have shown two potential characteristics of *de novo* centriole assembly: (1) the ratio of cells with more than two centrosomes after SAK overexpression in acentrosomal *Drosophila* S2 cells was significantly increased compared with the control; (2) *de novo* formed centrosomes, in *Drosophila* unfertilized eggs overexpressing SAK, displayed scatterred and random positions compared with the clustered phenotype of *de novo* formed centrosomes in SAK overexpressing embryos. Moreover, Peel et al. (2007) showed that induction of *de novo* centriole-like structures in unfertilized *Drosophila* eggs through overexpression of DSas-6, DSas-4 and SAK led to the formation of supernumerary centrioles.

Additionally, centrosome ablation studies in dividing mammalian cells, specifically in HeLa, RPE-1, HMEC and CHO culture cells, showed that *de novo* centriole assembly was always preceded by the formation of multiple centrioles within the cytoplasm of single

cells (from 2 up to 14 centriole-like structures) (Khodjakov et al., 2002; La Terra et al., 2005; Uetake et al., 2007).

Although the present work was carried out in *Drosophila* somatic cells which are not comparable either to unfertilized *Drosophila* eggs or to mammalian culture cells, the evidence from the previous studies plus the data showed here support the idea that centriole amplification and the loss of spatial restriction might potentially be considered two hallmarks of *de novo* centriole biogenesis that are transversal to different organisms and cell types. In fact, it has been advocated that the mother centriole might appear as an intermediate responsible for the spatial and numerical control of centriole biogenesis. In the absence of the parental centriole, the spatial restriction and the numerical control over daughter centriole formation are lost.

Recovery of the expression levels of centriolar proteins in *DSas-4 -/-* rescued cells

In spite of the existence of two modes of centriole assembly, canonical/template and *de novo*, there is evidence that the molecular machinery underlying both forms of centriole biogenesis is the same (A. Rodrigues-Martins et al., 2007b*;* Peel et al., 2007). Since *DSas-4 -/-* cells are only mutant for DSas-4 protein and given the previous evidence for the potential formation of centriole-like structures after DSas-4-GFP expression in *DSas-4 -/- R* cells, it became imperative to address if the conserved centriole assembly pathway was activated through the recovery in the expression levels of specific proteins associated with the centriole biogenesis process.

Asterless is a key element and upstream regulator of *Drosophila* centriole duplication (Dzhindzhev et al., 2010; Varmark et al., 2007). DSas-6 is a structural protein involved in the first steps of procentriole assemby and responsible for building the cartwheel in *Drosophila* and mammalian centrioles (Mennella et al., 2012; Rodrigues-Martins et al., 2007a; Van Breugel et al., 2011). As shown before, both proteins seemed to be either downregulated or delocalized in *DSas-4 -/-* mutant cell line (part 1, 1.2.), in which there was a decrease of approximately 70% of Asl levels at the centrosome and DSas-6 was absent in 100% of *DSas-4 -/-* cells.

A comparative analysis of the protein expression pattern by western blot for Asl and DSas-6 revealed a rescue in the protein expression levels in the *DSas-4 -/- R* cells compared with the mutant *DSas-4 -/-* cell line. Although protein quantification was not performed, it is clear that there was a significant increase in the amount of protein between the DSas-4 transfected and non-transfected cell lines, mainly for Asl, in which there was almost a rescue in the expression levels similar to control cells. This recovery in

the expression levels of specific centriolar proteins might be suggestive of an activation of the centriole assembly pathway after DSas-4 expression, and therefore, representing the activation of *de novo* centriole biogenesis process.

Interestingly, velocity sedimentation studies in *Drosophila* S2 cells overexpressing DSas-4 revealed that DSas-4-induced foci co-fractionate with several PCM components, including Asl (Gopalakrishnan et al., 2013). This evidence for a strong interaction between Asl and DSas-4 is in agreement with the dramatic increase verified for Asl levels in *DSas-4 -/-* rescued cell line.

As mentioned before, DSas-4 scaffolds cytoplasmic complexes during PCM formation and centrosome maturation. These S-CAP complexes include Asl and Cnn (Gopalakrishnan et al., 2012; Gopalakrishnan et al., 2013). Given this evidence, it is possible that the rescue in the protein expression levels of Asl can be associated with the formation of S-CAP complexes and are not directly related with the activation of the *de novo* centriole duplication process.

Strikingly, in contrast to the recovery in Asl and DSas-6 protein levels between transfected and mutant DSas-4 cells, a transcriptomic analysis performed in the brain and imaginal disks of *Drosophila* DSas-4 and DSas-6 mutant flies (lacking centrosomes) did not reveal a significant differential expression between both mutants. Only in DSas-4 mutants there was a mis-regulation (up or down-regulation) in 14 genes compared to the WT background. However, these differentially regulated genes could not be clustered into any obvious functional pathway such as the centriole biogenesis pathway (Baumbach et al., 2012).

Cnn is a PCM protein directly linked to PCM formation and, therefore responsible for controlling centrosome size (Conduit et al., 2010). Surprisingly, and in opposition to Asl and DSas-6 rescue, Cnn expression levels did not dramatically change between transfected and non-transfected DSas-4 cell lines. Given that an interaction between DSas-4, Asl and Cnn has been reported (Conduit et al., 2010; Gopalakrishnan et al., 2012; Gopalakrishnan et al., 2013), it would be expected a rescue in the Cnn expression levels similar to what was verified for Asl and DSas-6 in *DSas-4 -/- R* cells. The rescue in Cnn expression would be suggestive of either a recovery in centrosome functionality as a consequence of the *de novo* centriole assembly or the formation of S-CAP complexes without the production of centriolar structures.

However, in contrast with Asl and DSas-6 biological functions, it is important to emphasize that Cnn is a PCM component and it is not directly associated with the centriole biogenesis process. For this reason, Cnn expression levels might not change dramatically in the presence or absence of centrioles, but its recruitment to centrosomes might be affected. Indeed, the characterization of acentriolar *DSas-4 -/-* mutant cell line for

centriolar and centrosomal proteins revealed that Cnn was detected as disperse dots of variable size at the mitotic poles, suggestive of centrosome delocalization.

The *de novo* formed DSas-4-GFP foci are able to nucleate microtubules and to accumulate Centrosomin during mitosis

The centrosome is the main microtubule organizing centre in animal cells. In the first stages of mitosis, the centrosome increases in size through the accumulation of PCM components and begins to define the mitotic spindle through the generation of astral microtubules according to an "outside-inside" process, in order to promote correct spindle positioning and accurate chromosome segregation (Meunier et al., 2012). Centrioles are key elements in this process because are focal points for the accumulation of pericentriolar material. In fact, Bobinnec and co-workers (1998) showed that centriole disintegration in HeLa cells led to PCM unstability and dispersion along with the loss of well-define MT asters.

In this regard, it was adressed the functionality of DSas-4-GFP foci in *DSas-4 -/- R* cells for the production of astral microtubules, mitotic spindle morphology and the accumulation of the specific PCM component, Centrosomin.

First, it was shown that the *de novo* DSas-4-GFP foci formed in mitotic DSas-4 rescued cells were able to nucleate astral microtubules, which is suggestive of a restoration in the microtubule organizing capacity. Indeed, the mitotic spindle in some *DSas-4 -/- R* cells seemed to derive from opposite and focused DSas-4-GFP positive poles. The evidence for astral microtubule nucleation in *DSas-4 -/- R* cells is a strong indicator of centrosome presence due to the *de novo* centriole assembly.

Nonetheless, the microtubule fibers emanating from DSas-4-GFP foci in *DSas-4 -/- R* cells varied in shape as well as in number. These newly formed astral MTs were usually smaller and not so abundant comparing with the long and very well-defined MT asters emanating from the centrosomes of control cells. This last evidence suggests that the *de novo* formed DSas-4-GFP foci may represent structurally abnormal centrioles unable to fully recruit PCM and, therefore to proper restore centrosome function as MTOC. In agreement with this hypothesis, Peel et al. (2007) showed that, in unfertilized *Drosophila* eggs overexpressing recombinant centriole-specific proteins, only the very bright and larger structures were able to organize microtubules and to accumulate PCM markers such as Cnn and γ-tubulin. Although the previous study lacked electron microscopy analysis, it suggests the presence of abnormal *de novo* centrioles that are not able to organize and recruit PCM components in order to form a fully functional centrosome.

Moreover, it was previously shown that somatic 1182-4D acentriolar cells were able to recruit PCM components (e.g. γ-tubulin) to the polar regions. These PCM aggregates retained some MT nucleation capacity, although the resulting aster-like structures were 3-4 fold smaller than wild-type cells (Moutinho-Pereira et al., 2009). Thus, it is possible that the MT asters seen in *DSas-4 -/- R* cells might just represent PCM aggregates with some MT nucleation capacity, but lacking centrioles. Interestingly, astral MTs emanating from PCM aggregates were not clearly discernible in the *DSas-4 -/-* mutant cells (see part 1, 1.1. and 1.2.). It will be interesting in the future to measure and compare the size of the MT asters between control and *DSas-4 -/- R* cells, in order to address whether there was a rescue in the astral MT formation.

To further investigate the microtubule nucleating capacity of the *de novo* formed DSas-4-GFP foci, the recruitment of Cnn was analysed. As already mentioned, Cnn is highly recruited to centrosomes at the onset of mitosis and is a very important element for PCM organization and centrosome maturation (Conduit et al., 2010; Dobbelaere et al., 2008; Megraw et al., 1999; Vaizel-Ohayon et al., 1999). DSas-4-GFP foci in *DSas-4 -/-R* mitotic cells showed the presence of Cnn as a single and bright dot at the two opposite poles, whereas it was detected as small agregates of variable size at the mitotic poles of DSas-4 mutant background. This apparent strong and focalized Cnn presence at DSas-4-GFP foci is in contrast with the western blot analysis previoulsly described, in which there was not a recovery in Cnn expression levels after DSas-4-GFP expression in *DSas-4 -/- R* cells. However, it should be noted that the principle behind western blot is an analysis over the total amount of protein in the cell, and not the amount of protein in a particular cell compartment such as the centrosome. As previously claimed, and even though the fluorescence intensity protein levels of Cnn in *DSas-4 -/- R* cell line were not measured, it might be possible that Cnn expression is not altered between acentriolar and rescued *DSas-4 -/-* cells, but this protein is more efficiently recruited and concentrated at the poles due to the potential formation of *de novo* MTOCs in *DSas-4 -/- R* cells. In fact, since it was verified a decrease of approximately 70% in the Cnn protein fluorescence intensity levels in the mitotic poles of mutant DSas-4 cells, it would be interesting to investigate if there is a recovery of Cnn protein fluorescence intensity in the *DSas-4 -/- R* cell line.

In a canonical centrosome, MT anchoring and organization is only performed by the mother centriole due to the presence of distal and sub-distal appendages and PCM enrichment, which are hallmarks of mature centrioles (Avidor-Reiss et al., 2013; Brito et al., 2012; Cunha-Ferreira et al., 2009; Debec et al., 2010). Because *Drosophila* centrioles do not show obvious appendages, one interesting possibility would be that DSas-4-GFP foci correspond to *de novo* centrioles that are able to mature only with the acquisition of PCM, and thus perform astral MT nucleating activity. In this study, recombinant DSas-4-

GFP expression was induced over a period of 48h and *DSas-4 -/-* cells have a doubling time of approximately 36h. Therefore, since centrioles require approximately two cell cycles from their initial formation to fully mature and, even though the kinetics of *de novo* centriole biogenesis is slower than the canonical pathway (A. Rodrigues-Martins et al., 2007b; Khodjakov et al., 2002; La Terra et al., 2005; Marshall et al., 2001), the previous conditions would, in principle, favor not only the *de novo* centriole formation, but also centriole maturation. This issue remains to be answered.

Given there was some evidence for astral MT nucleation activity from DSas-4-GFP foci, the overall mitotic spindle morphology in *DSas-4 -/- R* cells was analysed, specifically the percentage of cells exhibiting a bipolar and focused mitotic spindle. Even though it was verified that approximately half of the rescued cells (50%) with DSas-4-GFP foci exhibited a WT-like mitotic spindle followed by the dual spindle morphology, the percentage of rescued cells exhibiting a WT-like spindle did not significantly increase comparing with the percentage of DSas-4 mutant cells exhibiting the same mitotic spindle morphology (45%). In fact, the percentage of cells in each spindle category (barrel, dual, non-polarized and hyperpolarized) was not significantly different between the transfected and mutant DSas-4 cell lines. The incapacity to rescue mitotic spindle morphology in *DSas-4 -/- R* cells might reflect incomplete centrosome maturation.

Although a MT depolymerizing assay was not performed in order to identify sites of MT assembly, live cell imaging analysis of Jupiter-GFP in *DSas-4 -/- R* cell line suggests that mitotic cells containing DSas-4-GFP foci at the poles progressively established the mitotic spindle through discrete astral MT nucleation in an "outside-inside" process. Nevertheless, we cannot exclude that centrosome-independent mechanisms for mitotic spindle assembly are also involved.

Interestingly, the spindle morphology of some dividing *DSas-4 -/- R* cells was still non-focused in spite of astral microtubule generation from DSas-4-GFP foci. This last evidence is in line with the imunofluorescence results for mitotic spindle morphology previously described, and reinforces the notion that some DSas-4-GFP foci might represent centriole-like structures that are not fully functional due to abnormalities in their structure or to different stages of centriole maturation that impair MT nucleation capacity. Additionally, it could also be that only one of the poles has formed *de novo* centrioles, explaining the assymetry in the spindle morphology of *DSas-4 -/- R* cells.

In agreement with the DSas-4-GFP amplification verified by fixed cell analysis, live imaging of rescued *DSas-4 -/-* cells also showed the formation of multiple DSas-4-GFP foci within the cytoplasm of interphasic cells. Consequently, mitotic *DSas-4 -/- R* assembled multipolar spindles with more than two DSas-4-GFP spots from which discrete MT bundles emerged. La Terra et al. (2005) have also shown that multipolar spindles are

produced when *de novo* centriole assembly is induced in acentriolar HeLa cells. Moreover, the EM reconstruction revealed an association between *de novo* formed centrioles and the spindle poles. Therefore, it might be that DSas-4-GFP foci correspond to centriole-like structures that are able to mature and function as an MTOC.

Furthermore, there is also some evidence for an increase in the length of mitosis that seems to be dependent on mitotic spindle morphology. *DSas-4 -/- R* cells presenting nearly focused spindles spent half of the time to complete mitosis comparing to cells with non-focused spindles. Indeed, it is known that cells without functional centrosomes spend more time (~ 2-3 times) in mitosis, especially in G2-M phases, in which a MT network needs to be generated and organized for chromosome segregation in metaphase. In the absence of centrioles, there is the lack of two specialized sources of microtubules at the poles and consequently, the formation of anastral spindles is mainly performed by mitotic chromatin in a potential kinetically slower “inside-outside” process (Basto et al., 2006; Lecland et al., 2013; Moutinho-Pereira et al., 2013; Sir et al., 2013). Although the previous studies did not address the relationship of anastral spindle configurations and mitotic duration, one might expect that cells with anastral non-focused spindle configurations take more time in mitosis due to the absence of focal points like centrosomes that guide mitosis in a more efficient way than the remaining MT assembly pathways. A more detailed analysis of the mitotic spindle geometry and mitotic timing should be performed. Furthermore, given that it is believed that centrosome accelarates mitotic spindle assembly, it would be interesting to compare the duration of mitosis between WT mitotic spindles and *DSas-4 -/-* rescued cells presenting nearly focused spindles, and assess if there is a rescue in mitotic timing. If that is the case, it would potentially represent the assembly of MTOCs due to the *de novo* centriole assembly.

de novo **centriole-like structures are formed in *DSas-4 -/-* rescued cells**

Drosophila centrioles are formed by the cartwheel, a structure composed by a central hub from which nine spokes radiate outward and are responsible for displaying the centriole 9-fold symmetry. This structure is connected to nine sets of microtubule doublets that decorate the outer surface of the centriole (Gonczy et al., 2012; Guichard et al., 2010; Guichard et al., 2013) (for a more complete description of centriole structure see introduction).

An electron microscopy analysis became imperative to address whether DSas-4-GFP foci represented centrioles, since they incorporate PCM as well as centriolar markers and seemed to organize astral MTs to a certain degree.

The EM analysis of asynchronous *DSas-4 -/-* rescued cells revealed that DSas-4-GFP foci represent *de novo* assembled centrioles. These centrioles have developed into recognizable and structurally normal centrioles, apparently composed by the carthweel and a centriolar MT wall. In just one case, there was the presence of a centriole-like structure surrounded by microtubules

Additionally, some cells showed clouds of electron-dense material lacking centrioles. This is in line with the hypothesis that some DSas-4-GFP foci might only be aggregates of PCM material (e.g. Asl, Cnn, D-PLP), since DSas-4 forms S-CAP complexes with pericentriolar proteins. Although there was no presence of clouds of electron-dense material surrounded by MTs in this EM analysis, it is known that PCM aggregates have some MT nucleation capacity, as previously described by Bobinnec et al. (1998) and Moutinho-Pereira et al. (2009), in which MT regrowth assays in vertebrate and *Drosophila* cells revealed the presence of small foci of PCM together with short MTs disseminated both within the cytoplasm and in the mitotic poles. Moreover, as already mentioned, DSas-4 overexpression can originate acentriolar MTOCs containing γ-tubulin (Dzhindzhev et al., 2010). Furthermore, these aggregates of pericentriolar material may also represent the earlier stages of *de novo* centriole biogenesis since this mode of centriole formation is potentially preceded by the production of clouds of PCM material that might provide an ideal environment to support centriole assembly (Khodjakov et al., 2002; La Terra et al., 2005). Thus, it is likely that some DSas-4 cells expressing DSas-4-GFP protein may lack a functional centrosome.

Thus far, all studies approaching *de novo* centriole biogenesis either in *Drosophila* or mammalian cells, reported the presence of centriole-like structures in different stages of maturation (Bobinnec et al., 1998; Khodjakov et al., 2002; La Terra et al., 2005; Loncarek et al., 2008). These centriole-like structures included several types of structural anomalies such as incomplete cylinders, distorted centriolar walls and different cylinder lengths. It was also reported the presence of electron dense amorphous clouds that did not contain centrioles at their core (La Terra et al., 2005). Interestingly, only one study have reported the *de novo* formation of structurally normal centrioles (Rodrigues-Martins et al., 2007b). Additionally, the previous study along with time lapse microscopy analysis in HeLa cells (La Terra et al., 2005) have also showed the presence of procentrioles closely associated with the *de novo* assembled centrioles, which suggests a switch from *de novo* mode of assembly to rounds of canonical duplication. In opposition, Peel et al. (2007) showed that *de novo* formed centriole-like structures were not capable of undergoing rounds of template-mediated duplication.

The EM analysis performed in this study revealed the absence of procentrioles next to the *de novo* formed centriole-like structures, even in the ones that were more

structurally complete. One possible explanation for the absence of template duplication once *de novo* centrioles are assembled is related with the abnormal structure of the formed centrioles that can impair centriole maturation, and therefore, disrupt the function of *de novo* centrioles as mother centrioles.

Furthermore, and since EM analysis was done in asynchronous cells, it might also be possible that *DSas-4 -/- R* cells did not have enough time to enter in a second cell cycle and to duplicate the *de novo* formed centrioles, even though DSas-4-GFP expression was induced during 48h. It should be noted that procentriole assemby occurs in interphase after the completion of a first cell cycle. Indeed, it was shown in HeLa cells, that *de novo* assembled centrioles were able to mature and enter new rounds of duplication in a normal fashion only during the second cell cycle (La Terra et al., 2005).

Moreover, another interesting feature verified through EM is the absence of multiple centriole-like structures *per* cell, as would be expected given the DSas-4-GFP amplification verified by immunofluorescence, and as it was previously shown by La Terra et al. (2005) and Khodjakov et al. (2000). In fact, all cells analysed so far in this study only exhibited one centriole-like structure.

Nevertheless, it should be noted that the EM analysis should be repeated in order to achieve more clear conclusions and a more refined characterization of these *de novo* assembled centriole-like structures in somatic *Drosophila* cells.

5. CONCLUSIONS

In the present study, it was shown that somatic acentriolar *Drosophila* cells are able to produce centrioles through the *de novo* mode of centriole duplication. Taking advantage of a naturally occuring acentriolar cellular line derived from DSas-4 mutant *Drosophila* flies (Basto et al., 2006; Lecland et al., 2013), this study showed that *de novo* centrioles are formed upon the rescue of these cells with a recombinant DSas-4 protein.

The characterization of these acentriolar cells by immunofluorescence analysis revealed the presence of PCM aggregates usually exhibiting a scattered localization at the mitotic poles of *DSas-4 -/-* mutant cells, whereas there was no polar recruitment of centriole-specific proteins. The mitotic spindle of *DSas-4 -/-* cells exhibited a wide variety of spindle configurations typical from anastral mitotic spindles generated by non-centrosomal MT assembly pathways with the total absence of astral MTs. This acentriolar mitotic cell phenotype is an indicative of the tight association between centrioles and centrosomes and reflects the consequences of centriole disruption on centrosome formation and function.

The rescue of DSas-4 mutant cells through DSas-4 protein introduction revealed a surprising recovery in the acentriolar mitotic phenotype. Using a variety of cellular and molecular approaches, it was shown a recovery in the expression levels of centriolar proteins tightly associated with the process of centriole duplication, as well as their localization at the mitotic poles along with DSas-4-GFP recombinant protein. These rescued cells exhibited discrete astral MT foci, PCM recruitment and presented an amplification of DSas-4-GFP foci. A more detailed analysis through electron microscopy revealed the presence of morphologically normal centriole-like structures as well as electron-dense clouds of PCM material lacking centrioles.

The *de novo* centriole assembly has been, as of yet, reported in somatic vertebrate cells and *Drosophila* unfertilized oocytes (Khodjakov et al., 2002; La Terra et al., 2005; Uetake et al., 2007; Rodrigues-Martins et al., 2007b; Peel et al., 2007). Although both systems have been very useful for dissecting the steps underlying centriole duplication, there are still some gaps associated with their used. *Drosophila* oocytes represent one of the few cellular systems in which centrioles are naturally lost in the course of development so that it has been extensively used to study the centriole biogenesis process. Nevertheless, oocytes are meiotic cells that contain enough proteins to make 2^{13} centriole pairs, whereby it may become difficult to extrapolate what happens in somatic cycling cells in which centriole-specific proteins seem to be tightly regulated. The second cellular model relies on the centriole or centrosome laser ablation approach to transiently create an acentriolar cell from a normal centriolar background, therefore not representing a native acentriolar system.

Therefore, until now, there was no model system to fill the gap of studying centriole biogenesis in normal physiological conditions in somatic cells. Thus, this study attempted to bridge the previous reports by taking advantage of the *DSas-4 -/-* acentriolar line as a model system to bring more information about the centriole assembly process.

The surprising evidence that acentriolar somatic *Drosophila* cells can produce *de novo* centrioles and restore microtubule nucleation capacity is in agreement with the previous studies on centriole biogenesis, and suggests that centriole biogenesis is a template-free process, as well as the *de novo* and the canonical or template modes of centriole duplication may be two independent pathways that rely on the same molecular machinery. Moreover, it seems that the *de novo* capacity of producing centrioles is not only restricted to specialized cases, but it may be a general phenomenon among different organisms and cell types. Thus, why do centrioles arise from a pre-existing centriole during cell duplication ? And what is the true role of the mother centriole on procentriole formation ?.

Over the past years, different hypothesis have been claimed regarding the dichotomy between these two modes of centriole assembly. This study showed one aspect that has been directly linked to the *de novo* mode of centriole biogenesis – centriole amplification. Given this evidence, along with the data from previous studies, it is possible to conclude that the older centriole is an important element to spatially restrict and numerically control centriole duplication in each individual cell cycle. This older centriole may possess a docking site on its proximal end that regulates, stabilizes and accelerates procentriole formation, whereby inhibiting the formation of more than one centriole *per* cell cycle.

Therefore, the role of the mother centriole may be based on inhibiting the *de novo* centriole assembly and thus, to set a limit on the number of produced centrioles in S phase. Furthermore, since previous reports showed that the kinetics of *de novo* centriole assembly is slower than the template mode, it is highly likely that the mother centriole accelerates the process of procentriole formation. Thus, as Rodrigues-Martins et al. (2007) previously claimed, the mother centriole might not act as a bona fide template but rather as a platform for regulatory proteins (e.g. SAK) involved in the centriole duplication process, offering a kinetic advantage over the *de novo* centriole biogenesis pathway. In the absence of this older centriole, there is the lack of a proper centriole assembly platform, and thus, the spatial regulation, the numerical control and the kinetics of centriole generation are lost or slowed down.

Moreover, as argued by Loncarek et al. (2008), the mature mother centriole may also provide and maintain a well defined and compact cloud of PCM that will ultimately have an impact over centriole number and on the precise location of centriole assembly.

Another interesting hypothesis that might be claimed on the light of the previous arguments is based on the sequential and concomitantly occurrence of these two centriole biogenesis pathways during centriole duplication. It is possible that centriole biogenesis in S phase is first preceded by the *de novo* formation of centriolar precursors within PCM clouds throughout the cytoplasm, that is followed by the attachment to the proximal-end of the resident centriole of only one of those percursors. This last step would offer a kinetic advantage and restrict centriole number in order to promote fidelity in mitosis.

One pertinent question that may arise considering the data from this study and from other studies is, why do the *de novo* assembled centrioles frequently exhibit a wide variety of intermediate morphological stages. One might expect that the mother centriole could act as a bona fide template ensuring the correct structural assembly of the new centriole. Indeed, the presence of the pre-existing centriole may partially ensure a correct centriole structure assembly and therefore, it may function as a monitoring mechanism of centriole morphology. Further studies will be required to clarify this issue.

The evidence for a common set of proteins regulating the template and the *de novo* modes of centriole assembly has favor the hypothesis that these two modes are not more than variations of a common pathway (Gopalakrishnan et al., 2013; Loncarek et al., 2007). Moreover, both pathways seem to be strictly linked to each other since the *de novo* mode of centriole assembly never occurs when a mature centriole is present (Loncarek et al., 2008; La Terra et al., 2005). One might expect that if these two pathways were different, the *de novo* centriole formation should occur even in the presence of the mother centriole.

Therefore, cells may take advantage of redundacy, so that there are two possible mechanisms or “roads” based on the same molecular elements working on the same goal – centriole assembly.

Indeed, as previously claimed, it might be possible an interaction between these pathways, in which centriole assembly is first preceded by the *de novo* assembly of procentriole structures followed by the template mode, in which there is the attachment of one of those previously assembled structures into the mature centriole. This last speculative scenario may ensure a spatial and number restriction of centriole assembly. Another possibility is that, even though there are two choices of how to produce centrioles, cells may take advantage of the mother centriole as a platform for the new centriole assembly because it may offer a more parsimonious advantage by offering an ideal environment for the successful new centriole formation in S phase, while ensuring fidelity on centrosome biogenesis. In this regard, there could be a competition between both pathways, in which the templated mechanism ultimately inhibits the *de novo* pathway on each cell duplication cycle.

Strikingly, there is still one specific question that remains to be answered: "If template centriole duplication provides far more precise control over centriole numbers, then why do so many organisms employ centriole *de novo* formation, in such critical events as early embryogenesis ?" (Loncarek et al., 2009). Probably, the most striking example of precision on *de novo* centriole assembly is given during mouse development. It is known that centrioles arise *de novo* and in a proper number during the 16-cell blastomere stage, which is in contrast with all the existing reports addressing *de novo* centriole biogenesis in all model systems, including the present study. The nature of this difference remains a mystery, but it may lie on different concentrations of regulatory elements implicated in the centriole duplication pathway.

Centrosome amplification is a well-known hallmark of cancer cells. The presence of extra centrosomes increases the occurrence of multipolar mitosis, which in turn might provide an ideal scenario for chromosome misegregation events followed by genomic instability (Nigg and Raff, 2009). Our observations that the *de novo* pathway promotes the formation of extra centrioles raises the possibility of a connection between this mode of centriole assembly and cancerous cells. Since centriole duplication is a very conserved process among vertebrates and invertebrates, the DSas-4 mutant cell line might provide an ideal system to address the previous issue.

To conclude, it is worth noting that the present study is a collection of still very preliminary data that needs to be consolidated. Nonetheless, it provides the proof-of-principle of the *de novo* centriole biogenesis process on somatic cycling cells under normal physiological conditions

Based on the expression “a picture is worth a thousand words”, this study gives very strong evidence for a template-free mechanism for centriole biogenesis. This study attempts to be a starting point or a groundwork to prompt more questions and to stimulate further studies.

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